

IMMUNITY AND CELL SIGNALING IN THE HOST RESPONSE TO
TOXOPLASMA GONDII INFECTION

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TOXOPLASMA GONDII INFECTION

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The immune response to an invading pathogen must be tightly regulated so as to combat the infection while avoiding immune-mediated pathologies. This requires the coordinated response of both innate and adaptive immunity, involving an array of cell types, including T cells, macrophages, and dendritic cells. While many studies have examined the general host immune response to infection, this thesis aimed to more clearly dissect the roles of the chemokine receptor CXCR3 and β -catenin in the coordination of this complex response by T cells and dendritic cells, respectively.

I show that CXCR3 expression is required on $CD4^+$ T cells for efficient clearance of the protozoan parasite *Toxoplasma gondii* during the intestinal response to infection. In the absence of this receptor, impaired $CD4^+$ T cell-dependent IFN- γ renders intestinal inflammatory monocytes inactive, and mice succumb to intestine-restricted overgrowth of parasite.

Tissue resident dendritic cells are the first immune cells to recognize foreign antigen and are crucial to the initiation of adaptive immunity. I show that differentiation of $CD8\alpha^+$ and related $CD103^+$ tissue resident DC subsets is positively regulated by β -catenin signaling, a pathway normally associated with tumorigenesis. Constitutive β -catenin signaling in DC thus leads to susceptibility of mice to *Toxoplasma gondii*

infection by excessively promoting proinflammatory DC subsets in dependence on interferon regulatory factor (IRF) 8 expression, resulting in hyperactivation of CD4⁺ T cells.

Interestingly, I show that β -catenin signaling in bone marrow-derived DC (BMDC) displays the reverse phenotype, whereby antigen-specific T cell activation and cytokine production are impaired by these DC. This finding underscores the phenotypic and genetic variation among different DC subsets and that β -catenin has the capacity to promote both inflammatory and tolerant DC subsets depending on context.

The data in this thesis provide novel insight into the mechanisms of immune cell trafficking, signaling, and differentiation, which ultimately lead to host protection against microbial insult.

BIOGRAPHICAL SKETCH

Sara was born on June 20th, 1985 in San Francisco, California to Steve and Elly Cohen. There, she spent her childhood years traipsing around the city with her sister Aliza, playing soccer, running cross country, and studying the classical guitar. Despite always having an interest in infection and how diseases worked, she pursued this more clearly in high school by taking microbiology and molecular genetics courses and reading books like *The Hot Zone* and *Demon in the Freezer*. She decided to then pursue a microbiology degree at Cornell University in Ithaca, NY. There she worked with Dr. Gregory Martin on *Pseudomonas* infection in tobacco as a Cornell Hughes Research Scholar, and she graduated in 2007 with a BA in Biological Sciences. During her time at Cornell, Sara developed a strong interest in parasitology and wanted to continue lab work in this general field. Following graduation, she applied to and was awarded a CDC/APHL Emerging Infectious Diseases fellowship to study vector-borne disease epidemiology at the Tennessee Department of Health in Nashville with Dr. Abelardo Moncayo. After a successful tenure in Nashville, Sara returned to Cornell University to continue her interest in disease pathogenesis and parasitology by pursuing a PhD in immunology with Dr. Eric Denkers studying *Toxoplasma gondii*. In her spare time, Sara loves to hike and bike, cook and bake delicious things, play the guitar and piano, sew, and spend time with friends.

To my family for their constant support and in loving memory of Bunk

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LIST OF ABBREVIATIONS

BSA, bovine serum albumin

BMDC, bone marrow-derived dendritic cell

CK, casein kinase

CD, cluster of differentiation

cDC, conventional dendritic cell

CFSE, 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester

DAPI, 4',5-diamidino-2-phenylindole I

DC, dendritic cell

DMSO, dimethylsulfoxide

ELISA, enzyme-linked immunosorbent assay

EDTA, ethylenediaminetetraacetic acid

ERK, extracellular signal-regulated kinase

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GM-CSF, granulocyte-monocyte colony stimulating factor

GSK, glycogen synthase kinase

H&E, hematoxylin and eosin

HRP, horse radish peroxidase

IBD, inflammatory bowel disease

i.p., intraperitoneal

IFN, interferon

iNOS, inducible nitric oxide synthase

IL, interleukin

IRF, interferon regulatory factor

IRG, immunity-related GTPase

KO, knockout

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MFI, median fluorescent intensity

MHC, major histocompatibility complex

MLN, mesenteric lymph node

moDC, monocyte-derived dendritic cell

mRNA, messenger ribonucleic acid

NK, natural killer

NO, nitric oxide

OCT, optimal cutting temperature

OVA, ovalbumin

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PEC, peritoneal exudate cells

pDC, plasmacytoid dendritic cell

PMA, phorbol 12-myristate 13-acetate

PP, Peyer's patch

qPCR, quantitative polymerase chain reaction

RNA, ribonucleic acid

STAg, soluble tachyzoite antigen

STAT, signal-transducer and activator of transcription

Tcf/Lef, T cell factor/Lymphoid enhancer factor

Th1, T helper 1

TNF, tumor necrosis factor

Treg, regulatory T cell

LIST OF SYMBOLS

α : alpha

β : beta

$^{\circ}$: degrees

γ : gamma

μ : micro

CHAPTER 1

**Introduction to *Toxoplasma gondii*, monocytes, dendritic cells,
and Wnt signaling**

Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular protozoan parasite within the Apicomplexa phylum. It was simultaneously discovered by Charles Nicolle and Louis Manceaux in Tunisia and by Alfonso Splendore in Brazil in 1908 (1). It was ultimately discovered that *T. gondii* sexual reproduction can occur only in the intestinal tract of members of the cat family, and all other hosts, including rodents and people, are intermediate hosts (Figure 1.1) (2-4). As major prey of cats, rodents are a primary natural intermediate host of *T. gondii*, and they therefore serve as excellent experimental models for its study. Among people, seropositivity of *T. gondii* varies by region from 10-80%, but within the United States it nears 11% (5, 6). Most infections are asymptomatic, but in immunocompromised individuals, particularly AIDS patients, infection can be fatal. The most common symptom is encephalitis, and during the peak of the AIDS epidemic in the early 1990s, an estimated 10% of patients in the US died annually from this complication (7). However, the number of AIDS-associated toxoplasmic encephalitis cases dropped 3-fold to only 3,643 cases by 2001 as a result of improved anti-retroviral therapies (8).

Toxoplasmosis may also be acquired congenitally if the mother becomes infected for the first time during pregnancy. The earlier in pregnancy the infection occurs, the more severe the disease outcome, with the most common symptoms being retinochoroiditis, hydrocephalus, and ocular disease. The other major routes of transmission are ingestion of tissue cysts from undercooked meat, oocysts from food or water contaminated by cat feces, or organ transplants and blood transfusions from infected donors (5). Within immunocompetent hosts, the most frequent symptom is

lymphadenopathy, and infection generally goes unnoticed. *T. gondii* has many features in common with other disease-causing protozoans, including *Plasmodium*, *Trypanosoma*, and *Leishmania* (9). Therefore, understanding the immune response of a ubiquitous and potentially fatal pathogen such as *T. gondii* can provide insight into the immune response to other diseases of public health importance.

T. gondii is the sole species within the *Toxoplasma* genus and is sorted into 3 distinct clonal lineages based on allelic variation. In mice, these lineages vary in virulence, with type I being the most virulent and types II and III being less virulent (10). *T. gondii* is unique among other Apicomplexans in that it can infect any warm-blooded animal. Upon ingestion of oocysts or tissue cysts, sporozoites or bradyzoites, respectively, excyst and penetrate the intestinal epithelium, converting into fast replicating tachyzoites (11). Cell invasion by tachyzoites is an active process involving the parasite secretory organelles micronemes, rhoptries, and dense granules. During invasion, these organelles release their contents to form a moving junction with the host plasma membrane, where they then enter the host cell and manipulate host signaling cascades (12-18). The parasite resides in the subsequently formed parasitophorous vacuole (PV) that excludes all but GPI-anchored host proteins. This structure acts as a molecular sieve by allowing passive diffusion of molecules ≤ 1400 Daltons for nutrient acquisition, yet protecting *T. gondii* from degradation as it remains segregated from host endocytic and exocytic pathways (19, 20). The timing and location of parasitophorous vacuole formation appears to be strain-specific, as Type I parasites form a moving junction at the plasma membrane upon active host cell

invasion, whereas avirulent Type II parasites form a moving junction from within the phagosome following phagocytic uptake (21).

General immune response to *Toxoplasma gondii*

Toxoplasma mounts a potent cell-mediated immune response that involves the cooperation of multiple components of the host immune system. Unless the host is immunocompromised upon infection, this immune response is sufficient to protect from overt disease, and the parasite is forced into a quiescent cyst stage, where it can remain for the duration of the life of the host (22). Protective immunity is dependent on the proinflammatory cytokines IL-12 and IFN- γ , as the absence of either can lead to rapid death of the host at the acute phase of *T. gondii* infection (23, 24). IL-12 can be produced by dendritic cells, macrophages, and neutrophils (25-28), and this IL-12 serves to activate a Th1 program in CD4⁺ and CD8⁺ T cells as well as natural killer (NK) cells, leading to the secretion of high levels of IFN- γ (Figure 1.2) (22, 29-31).

The requirement for IFN- γ in host defense against *T. gondii* has long been recognized (32). Early studies identified a role for IFN- γ in activating microbicidal functions in macrophages, such as upregulation of reactive oxygen intermediates and the tryptophan degrading enzyme indoleamine dioxygenase (IDO), leading to parasite elimination in vitro and during chronic infection (33, 34). More recent studies argue that IFN- γ signaling activates immunity-related GTPases (IRG) that disrupt the parasitophorous vacuole in which the parasite resides, leading to its elimination from the host cell (35-37). Interestingly, IRG proteins are not expressed by humans, leading to fascinating questions about the undetermined role of IFN- γ in host protection

against *T. gondii* in humans as well the evolutionary relationships between *Toxoplasma*, humans, and other intermediate hosts of infection (38).

The natural route of *Toxoplasma* infection is peroral, and the initial site of infection is therefore the gut. The mucosal immune response is specially equipped to combat such infections, and the nature of this response is discussed in detail in Chapter 2. Importantly, two of the first cell types to recognize and respond to *T. gondii* are those lying within the monocyte/macrophage and dendritic cell lineages.

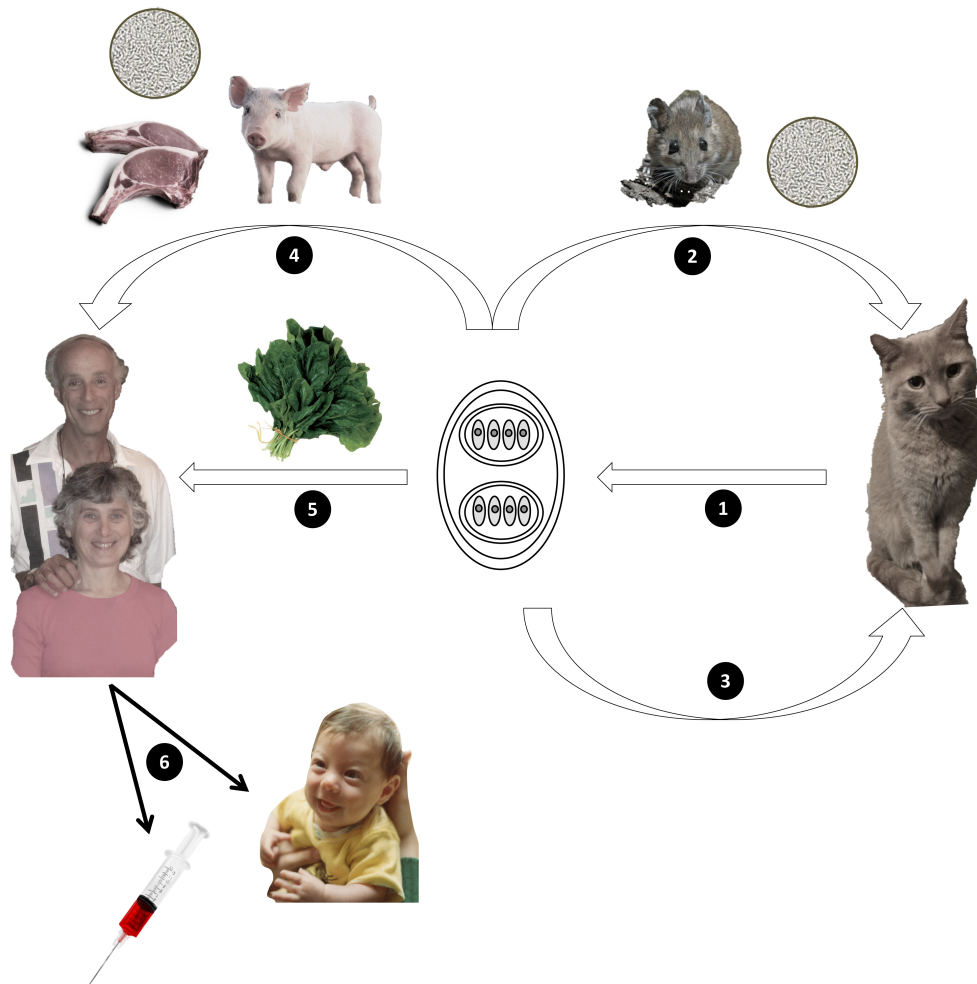


Figure 1.1 Life cycle of *Toxoplasma gondii*. (1) Members of the cat family shed oocysts through their feces, and these oocysts sporulate in the environment to become infectious, each containing 2 sporocysts with 4 sporozoites. Intermediate hosts, such as rodents, ingest these cysts in the environment, releasing tachyzoites that convert to life-long tissue cysts. Cats can become reinfected through (2) carnivorousism of rodents harboring tissue cysts or (3) through direct ingestion of sporulated oocysts, and sexual reproduction of the parasite can again occur within the feline small intestine. Humans are incidental hosts that can be infected through (4) ingestion of undercooked meat from intermediate hosts harboring tissue cysts, such as pork products, or (5) ingestion of vegetable products that have been contaminated with oocysts. (6) Other mechanisms of human transmission include vertical transmission from infected pregnant mother to fetus and horizontal transmission via donation of contaminated blood or tissue, such as during blood transfusion or organ transplantation.

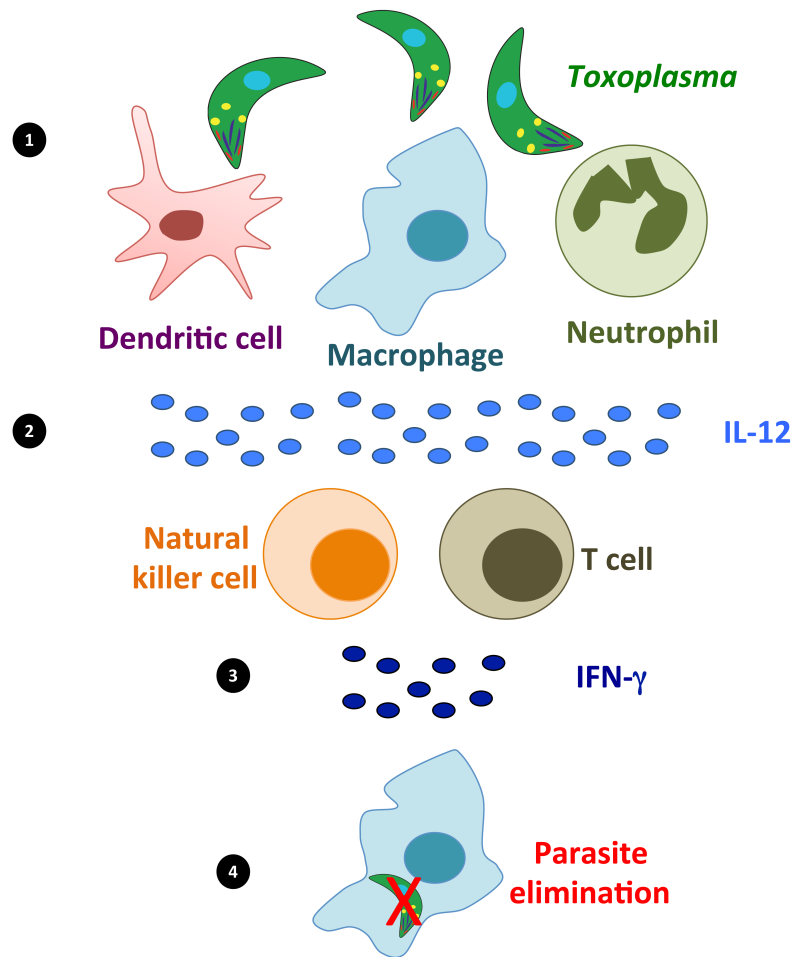


Figure 1.2 General immune response to *Toxoplasma gondii*. (1) Initial parasite recognition is mediated by innate immune cells, including dendritic cells, macrophages, and neutrophils. (2) TLR-dependent recognition of parasite results in the secretion of IL-12. (3) IL-12 activates natural killer cells and T cells to produce IFN- γ . (4) IFN- γ signaling is key for the activation of antimicrobial effector cells, including inflammatory monocytes, which eliminate the parasite via mechanisms including reactive oxygen intermediates, immunity-related GTPase induction, and tryptophan degradation.

Role of monocytes and macrophages during *Toxoplasma* infection

Blood monocytes emerge from the bone marrow as precursors of macrophages and are recruited to tissues in response to infection-induced chemokines, where they take on the name inflammatory monocyte and are distinguished by expression of Ly6C, TNF- α , and iNOS (Figure 1.3) (39). They are frequently infected in vivo upon *T. gondii* infection and are a source of protective IL-12 (40-42). Furthermore, they are required for protection against *T. gondii*, as mice with impaired emigration of monocytes from the bone marrow are highly susceptible to infection (41, 43). *T. gondii* has acquired multiple mechanisms to subvert macrophage function, including inhibition of cytokine production, altered chromatin remodeling at IFN- γ -responsive promoters, and inactivation of IRG molecules (12, 13, 44, 45). Macrophages clearly play a significant role in immunity during *T. gondii* infection, and further details regarding this role are discussed in Chapter 2.

Dendritic cell subsets and function

Dendritic cells (DC) are unique innate immune cells that, in addition to recognizing and responding to foreign antigen, initiate adaptive immunity through antigen presentation (46). DC originate from a common dendritic progenitor in the bone marrow and can later be subdivided into tissue-resident conventional DC (cDC), plasmacytoid DC (pDC), dermal-resident Langerhans DC, and monocyte-derived DC (moDC). These subsets are distinguished by surface marker expression, transcription and growth factor requirements, tissue distribution, and function (Figure 1.3). For example, splenic CD8 α^+ DC depend on IRF8, Id2, Batf3, and Nfil3 and are

considered efficient cross-presenting DC (47-51), while splenic CD4⁺ DC require IRF4 expression and more effectively activate MHCII-restricted CD4⁺ T cells (52). Conversely, pDC are poor antigen presenters yet secrete high levels of type I IFN following viral infection, and they require IRF8, STAT3, and E2-2 for differentiation (53, 54).

The CD8 α ⁺ DC subset is particularly crucial for the immune response to *Toxoplasma gondii* infection, as genetic deletions of CD11c or the CD8 α ⁺ DC transcription factor Batf3 result in susceptibility to infection associated with impaired IL-12 secretion (55-57). Details of the role of DC during toxoplasmosis are discussed in Chapter 2.

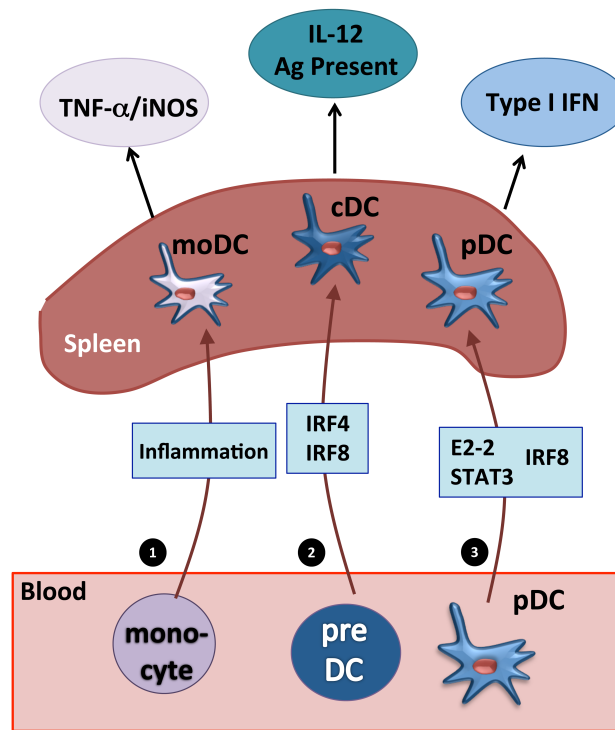


Figure 1.3 Differentiation and function of dendritic cell subsets and macrophages.

(1) Monocyte-derived DC (moDC) differentiate from blood monocytic precursors following inflammatory signals that recruit them into the tissue. There they upregulate $\text{TNF-}\alpha$ and iNOS and are key effectors against intracellular pathogens. (2) Precursors of DC (pre-DC) upregulate transcription factors upon entry into tissues, including interferon regulatory factors (IRF) 4 and 8, which direct conventional DC (cDC) subsets that antigen present and produce IL-12. (3) Plasmacytoid DC (pDC) differentiate directly from common dendritic progenitors in the bone marrow based on expression of transcription factors E2-2, STAT3, and IRF8, and they are key responders to viral infections due to their strong capacity to secrete type I IFN.

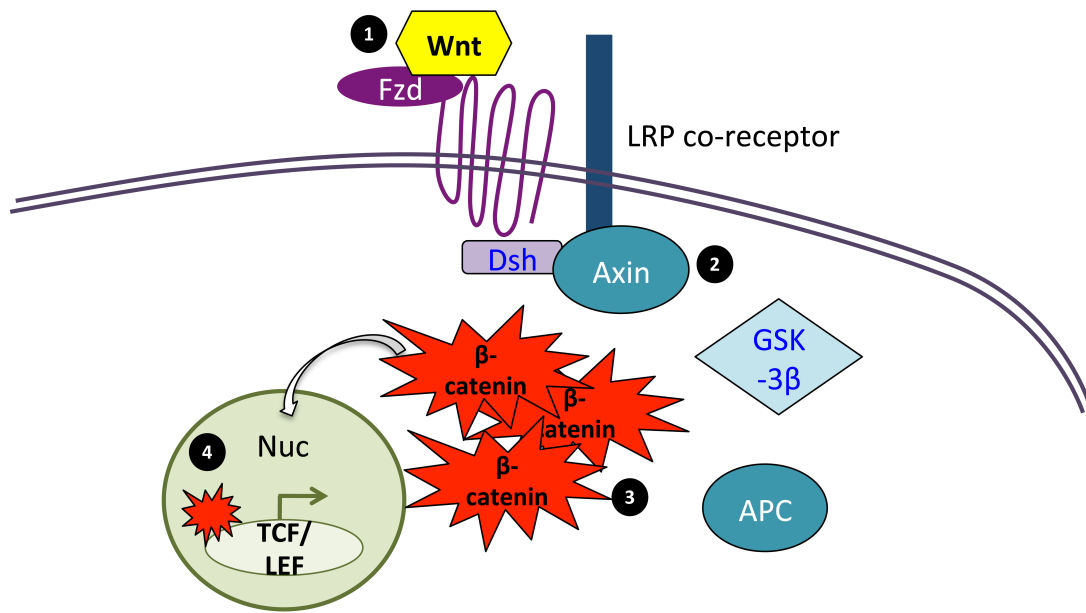


Figure 1.4 Overview of the Wnt signaling pathway. The Wnt pathway centers around the transcription factor β -catenin, which is normally phosphorylated by a complex containing glycogen synthase kinase (GSK)-3 β , Axin, and adenomatous polyposis coli (APC), leading to its proteasomal degradation. (1) Upon Wnt ligand binding to Frizzled (Fzd) receptors, phosphorylation of β -catenin is inhibited due to recruitment of Axin to the low density lipoprotein receptor-related protein (LRP) at the cell membrane, thus (2) disassociating the destruction complex. (3) β -catenin then accumulates in the cytoplasm and subsequently translocates to the nucleus. (4) There, it activates a wide array of transcriptional programs in conjunction with its co-factors T cell factor/lymphoid enhancer factor (TCF/LEF).

Role of Wnt signaling in dendritic cell programming

Wnt signaling is an essential physiological pathway, as its deletion results in embryonic lethality, and activating mutations are often oncogenic (58, 59). The Wnt pathway centers around the transcription factor β -catenin, whose cytoplasmic levels are tightly regulated by continual phosphorylation, which results in its proteasomal degradation. Wnt ligand binding to cell surface Frizzled receptors inhibits this phosphorylation, resulting in the cytoplasmic accumulation and nuclear translocation of β -catenin, where it activates an array of target genes (Figure 1.4). Example transcriptional targets of β -catenin include genes related to proliferation, apoptosis, survival, and stem cell renewal (60-62). More recently, Wnt signaling has been recognized for its role in hematopoiesis and immunity (63), and this role has been extended to dendritic cell development and function. Activating Wnt signals during DC development from bone marrow precursors can enhance DC differentiation and maturation (64), and maturation of bone marrow-derived DC (BMDC) by cluster disruption activates β -catenin signaling, promoting an anti-inflammatory phenotype that is protective against experimental autoimmune encephalitis (EAE) (65). Furthermore, genetic deletion of β -catenin from CD11c⁺ cells results in the emergence of a proinflammatory phenotype in small intestinal T cells, suggesting a role for β -catenin in promoting tolerance in the intestine (66). Therefore, it has become increasingly accepted that β -catenin signaling plays a role in controlling DC function both in vivo and in vitro, and the consensus is that β -catenin promotes tolerizing signals to DC.

Outline of dissertation research

The host immune response to infection requires a tightly regulated balance between the generation of proinflammatory cells to combat invading pathogens and anti-inflammatory cells to prevent potentially harmful immune activation. The simultaneous coordination of these events is a highly complex process involving cells of both the innate and adaptive arms of the immune system. The aim of this dissertation was to examine several of these facets of host immunity both during homeostasis as well as during infection with the protozoan parasite *Toxoplasma gondii*.

Chapter 2 provides a discussion of the currently available data regarding the intestinal immune response following oral infection with *T. gondii*. In line with mucosal immunity, **Chapter 3** assesses the role of chemokine receptor CXCR3 in the establishment of protective immunity against *T. gondii* following natural peroral infection. This study identifies CXCR3 as required for the optimal trafficking of CD4⁺ T cells to the small intestine to secrete IFN- γ , thus activating inflammatory monocytes to eliminate parasite in the gut. In the absence of CXCR3, mice succumb at the acute phase of infection in association with overgrowth of parasite in the small intestine, impaired T cell IFN- γ and monocyte IL-12, and influx of pathogenic TNF- α -secreting neutrophils to the gut.

My interest in β -catenin stemmed from the finding that it promotes tolerance in the intestine (66), and I hypothesized that constitutive DC β -catenin signaling would protect mice from the lethal inflammatory bowel-like phenotype that results from high dose *T. gondii* infection (67). As seen in **Chapter 4**, mice with constitutive β -catenin

signaling in DC are equally susceptible to high dose infection and are more susceptible to low dose infection. This susceptibility phenotype is due to β -catenin-dependent IRF8 upregulation, leading to the expansion of splenic CD8 α^+ DC that hypersecrete IL-12 upon stimulation with *Toxoplasma* or LPS despite increased regulatory T cell induction. This study identifies a novel role for β -catenin in tissue resident DC differentiation and activation.

In **Chapter 5**, the effect of β -catenin signaling on BMDC and tissue resident splenic DC was compared. Interestingly, BMDC acquired a largely anti-inflammatory phenotype upon β -catenin activation, as IL-12 secretion was reduced following stimulation, and antigen-specific BMDC-dependent CD4 $^+$ T cell proliferation was strongly impaired. We conclude that different DC subsets are differentially affected by β -catenin signaling, potentially due to differences in the availability of target gene promoters or transcription factors.

Finally, the significance of these findings and how they serve to better our understanding of host immunity in the face of microbial infection is described in **Chapter 6**. Future directions for continuation of these projects are also discussed.

References

1. Dubey, J. P. 2008. The history of *Toxoplasma gondii*--the first 100 years. *J. Eukaryot. Microbiol.* 55: 467–475.
2. Hutchison, W. M. 1965. Experimental transmission of *Toxoplasma gondii*. *Nature* 206: 961–962.
3. Frenkel, J. K., J. P. Dubey, and N. L. Miller. 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. *Science* 167: 893–896.
4. Dubey, J. P., N. L. Miller, and J. K. Frenkel. 1970. The *Toxoplasma gondii* oocyst from cat feces. *J. Exp. Med.* 132: 636–662.
5. Hill, D., and J. P. Dubey. 2002. *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin. Microbiol. Infect.* 8: 634–640.
6. Jones, J. L., D. Kruszon-Moran, K. Sanders-Lewis, and M. Wilson. 2007. *Toxoplasma gondii* infection in the United States, 1999–2004, decline from the prior decade. *Am. J. Trop. Med. Hyg.* 77: 405–410.
7. Luft, B. J., and J. S. Remington. 1992. Toxoplasmic encephalitis in AIDS. *Clin. Infect. Dis.* 15: 211–222.
8. Jones, J. L., and J. M. Roberts. 2012. Toxoplasmosis hospitalizations in the United States, 2008, and trends, 1993–2008. *Clin. Infect. Dis.* 54: e58–61.
9. Gazzinelli, R. T., and E. Y. Denkers. 2006. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol* 6: 895–906.
10. Howe, D. K., and L. D. Sibley. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J. Infect. Dis.* 172: 1561–1566.
11. Dubey, J. P., D. S. Lindsay, and C. A. Speer. 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin. Microbiol. Rev.* 11: 267–299.
12. Butcher, B. A., B. A. Fox, L. M. Rommereim, S. G. Kim, K. J. Maurer, F. Yarovinsky, D. R. Herbert, D. J. Bzik, and E. Y. Denkers. 2011. *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog* 7: e1002236.
13. Schneider, A. G., D. S. Abi Abdallah, B. A. Butcher, and E. Y. Denkers. 2013. *Toxoplasma gondii* triggers phosphorylation and nuclear translocation of dendritic cell

STAT1 while simultaneously blocking IFN γ -induced STAT1 transcriptional activity. *PLoS ONE* 8: e60215.

14. Rosowski, E. E., D. Lu, L. Julien, L. Rodda, R. A. Gaiser, K. D. C. Jensen, and J. P. J. Saeij. 2011. Strain-specific activation of the NF-kappaB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. *Journal of Experimental Medicine* 208: 195–212.

15. Denkers, E. Y., L. Kim, and B. A. Butcher. 2003. In the belly of the beast: subversion of macrophage proinflammatory signalling cascades during *Toxoplasma gondii* infection. *Cell. Microbiol.* 5: 75–83.

16. Carruthers, V., and J. C. Boothroyd. 2007. Pulling together: an integrated model of *Toxoplasma* cell invasion. *Curr. Opin. Microbiol.* 10: 83–89.

17. Melo, M. B., K. D. C. Jensen, and J. P. J. Saeij. 2011. *Toxoplasma gondii* effectors are master regulators of the inflammatory response. *Trends Parasitol* 27: 487–495.

18. Denkers, E. Y., D. J. Bzik, B. A. Fox, and B. A. Butcher. 2012. An inside job: hacking into Janus kinase/signal transducer and activator of transcription signaling cascades by the intracellular protozoan *Toxoplasma gondii*. *Infection and Immunity* 80: 476–482.

19. Mordue, D. G., N. Desai, M. Dustin, and L. D. Sibley. 1999. Invasion by *Toxoplasma gondii* establishes a moving junction that selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring. *J. Exp. Med.* 190: 1783–1792.

20. Mordue, D. G., S. Håkansson, I. Niesman, and L. D. Sibley. 1999. *Toxoplasma gondii* resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. *Exp. Parasitol.* 92: 87–99.

21. Zhao, Y., A. H. Marple, D. J. P. Ferguson, D. J. Bzik, and G. S. Yap. 2014. Avirulent strains of *Toxoplasma gondii* infect macrophages by active invasion from the phagosome. *Proc. Natl. Acad. Sci. U.S.A.*

22. Sher, A., E. Y. Denkers, and R. T. Gazzinelli. 1995. Induction and regulation of host cell-mediated immunity by *Toxoplasma gondii*. *Ciba Found. Symp.* 195: 95–104–discussion 104–9.

23. Scharton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, S. Hieny, R. T. Gazzinelli, and A. Sher. 1996. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to *Toxoplasma gondii* while failing to control acute infection. *J. Immunol.* 157: 4045–4054.

24. Ely, K. H., L. H. Kasper, and I. A. Khan. 1999. Augmentation of the CD8+ T cell response by IFN-gamma in IL-12-deficient mice during *Toxoplasma gondii* infection. *J. Immunol.* 162: 5449–5454.
25. Bliss, S. K., Y. Zhang, and E. Y. Denkers. 1999. Murine neutrophil stimulation by *Toxoplasma gondii* antigen drives high level production of IFN-gamma-independent IL-12. *J. Immunol.* 163: 2081–2088.
26. Kim, L., L. Del Rio, B. A. Butcher, T. H. Mogensen, S. R. Paludan, R. A. Flavell, and E. Y. Denkers. 2005. p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection. *J. Immunol.* 174: 4178–4184.
27. Reis e Sousa, C., S. Hieny, T. Scharton-Kersten, D. Jankovic, H. Charest, R. N. Germain, and A. Sher. 1997. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J. Exp. Med.* 186: 1819–1829.
28. Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher. 1994. Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. *J. Immunol.* 153: 2533–2543.
29. Denkers, E. Y., R. T. Gazzinelli, D. Martin, and A. Sher. 1993. Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to *Toxoplasma gondii* in MHC class I-deficient mice. *J. Exp. Med.* 178: 1465–1472.
30. Sher, A., I. P. Oswald, S. Hieny, and R. T. Gazzinelli. 1993. *Toxoplasma gondii* induces a T-independent IFN-gamma response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor-alpha. *J. Immunol.* 150: 3982–3989.
31. Dupont, C. D., D. A. Christian, and C. A. Hunter. 2012. Immune response and immunopathology during toxoplasmosis. *Semin Immunopathol* 34: 793–813.
32. Suzuki, Y., M. A. Orellana, R. D. Schreiber, and J. S. Remington. 1988. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* 240: 516–518.
33. Jun, C. D., S. H. Kim, C. T. Soh, S. S. Kang, and H. T. Chung. 1993. Nitric oxide mediates the toxoplasmastatic activity of murine microglial cells in vitro. *Immunol. Invest.* 22: 487–501.
34. Pfefferkorn, E. R. 1984. Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc. Natl. Acad. Sci. U.S.A.* 81: 908–912.

35. Zhao, Y. O., A. Khaminets, J. P. Hunn, and J. C. Howard. 2009. Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFN γ -inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. *PLoS Pathog* 5: e1000288.
36. Yamamoto, M., M. Okuyama, J. S. Ma, T. Kimura, N. Kamiyama, H. Saiga, J. Ohshima, M. Sasai, H. Kayama, T. Okamoto, D. C. S. Huang, D. Soldati-Favre, K. Horie, J. Takeda, and K. Takeda. 2012. A cluster of interferon- γ -inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. *Immunity* 37: 302–313.
37. Khaminets, A., J. P. Hunn, S. Könen-Waisman, Y. O. Zhao, D. Preukschat, J. Coers, J. P. Boyle, Y.-C. Ong, J. C. Boothroyd, G. Reichmann, and J. C. Howard. 2010. Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell. Microbiol.* 12: 939–961.
38. Gazzinelli, R. T., R. Mendonça-Neto, J. Lilue, J. Howard, and A. Sher. 2014. Innate resistance against *Toxoplasma gondii*: an evolutionary tale of mice, cats, and men. *Cell Host Microbe* 15: 132–138.
39. Shi, C., and E. G. Pamer. 2011. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* 11: 762–774.
40. Gregg, B., B. C. Taylor, B. John, E. D. Tait Wojno, N. M. Girgis, N. Miller, S. Wagage, D. S. Roos, and C. A. Hunter. 2013. Replication and distribution of *Toxoplasma gondii* in the small intestine after oral infection with tissue cysts. *Infection and Immunity* 81: 1635–1643.
41. Dunay, I. R., R. A. Damatta, B. Fux, R. Presti, S. Greco, M. Colonna, and L. D. Sibley. 2008. Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. *Immunity* 29: 306–317.
42. Cohen, S. B., K. J. Maurer, C. E. Egan, S. Oghumu, A. R. Satoskar, and E. Y. Denkers. 2013. CXCR3-dependent CD4⁺ T cells are required to activate inflammatory monocytes for defense against intestinal infection. *PLoS Pathog* 9: e1003706.
43. Dunay, I. R., A. Fuchs, and L. D. Sibley. 2010. Inflammatory monocytes but not neutrophils are necessary to control infection with *Toxoplasma gondii* in mice. *Infection and Immunity* 78: 1564–1570.
44. Fentress, S. J., M. S. Behnke, I. R. Dunay, M. Mashayekhi, L. M. Rommereim, B. A. Fox, D. J. Bzik, G. A. Taylor, B. E. Turk, C. F. Lichti, R. R. Townsend, W. Qiu, R. Hui, W. L. Beatty, and L. D. Sibley. 2010. Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii*-secreted kinase promotes macrophage survival and virulence. *Cell Host Microbe* 8: 484–495.

45. Leng, J., B. A. Butcher, C. E. Egan, D. S. Abi Abdallah, and E. Y. Denkers. 2009. Toxoplasma gondii prevents chromatin remodeling initiated by TLR-triggered macrophage activation. *The Journal of Immunology* 182: 489–497.
46. Mellman, I., and R. M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106: 255–258.
47. Hildner, K., B. T. Edelson, W. E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, R. D. Schreiber, T. L. Murphy, and K. M. Murphy. 2008. Batf3 deficiency reveals a critical role for CD8 α ⁺ dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097–1100.
48. Jackson, J. T., Y. Hu, R. Liu, F. Masson, A. D'Amico, S. Carotta, A. Xin, M. J. Camilleri, A. M. Mount, A. Kallies, L. Wu, G. K. Smyth, S. L. Nutt, and G. T. Belz. 2011. Id2 expression delineates differential checkpoints in the genetic program of CD8 α ⁺ and CD103⁺ dendritic cell lineages. *EMBO J.* 30: 2690–2704.
49. Kashiwada, M., N.-L. L. Pham, L. L. Pewe, J. T. Harty, and P. B. Rothman. 2011. NFIL3/E4BP4 is a key transcription factor for CD8 α ⁺ dendritic cell development. *Blood* 117: 6193–6197.
50. Aliberti, J., O. Schulz, D. J. Pennington, H. Tsujimura, C. Reis e Sousa, K. Ozato, and A. Sher. 2003. Essential role for ICSBP in the in vivo development of murine CD8 α ⁺ dendritic cells. *Blood* 101: 305–310.
51. Pooley, J. L., W. R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8⁺ dendritic cells, but cross-presented to CD8 T cells by CD8⁺ dendritic cells. *J. Immunol.* 166: 5327–5330.
52. Mount, A. M., C. M. Smith, F. Kupresanin, K. Stoermer, W. R. Heath, and G. T. Belz. 2008. Multiple dendritic cell populations activate CD4⁺ T cells after viral stimulation. *PLoS ONE* 3: e1691.
53. Li, H. S., C. Y. Yang, K. C. Nallaparaju, H. Zhang, Y.-J. Liu, A. W. Goldrath, and S. S. Watowich. 2012. The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development. *Blood* 120: 4363–4373.
54. Reizis, B., M. Colonna, G. Trinchieri, F. Barrat, and M. Gilliet. 2011. Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nat Rev Immunol* 11: 558–565.
55. Liu, C.-H., Y.-T. Fan, A. Dias, L. Esper, R. A. Corn, A. Bafica, F. S. Machado, and J. Aliberti. 2006. Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against Toxoplasma gondii infection in mice. *J. Immunol.* 177: 31–35.

56. Meredith, M. M., K. Liu, G. Darrasse-Jeze, A. O. Kamphorst, H. A. Schreiber, P. Guernonprez, J. Idoyaga, C. Cheong, K.-H. Yao, R. E. Niec, and M. C. Nussenzweig. 2012. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *Journal of Experimental Medicine* 209: 1153–1165.
57. Mashayekhi, M., M. M. Sandau, I. R. Dunay, E. M. Frickel, A. Khan, R. S. Goldszmid, A. Sher, H. L. Ploegh, T. L. Murphy, L. D. Sibley, and K. M. Murphy. 2011. CD8 α (+) dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites. *Immunity* 35: 249–259.
58. Haegel, H., L. Larue, M. Ohsugi, L. Fedorov, K. Herrenknecht, and R. Kemler. 1995. Lack of beta-catenin affects mouse development at gastrulation. *Development* 121: 3529–3537.
59. Harada, N., Y. Tamai, T. Ishikawa, B. Sauer, K. Takaku, M. Oshima, and M. M. Taketo. 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* 18: 5931–5942.
60. Biechele, T. L., R. M. Kulikaukas, R. A. Toroni, O. M. Lucero, R. D. Swift, R. G. James, N. C. Robin, D. W. Dawson, R. T. Moon, and A. J. Chien. 2012. Wnt/ β -catenin signaling and AXIN1 regulate apoptosis triggered by inhibition of the mutant kinase BRAFV600E in human melanoma. *Sci Signal* 5: ra3.
61. Masckauchán, T. N. H., C. J. Shawber, Y. Funahashi, C.-M. Li, and J. Kitajewski. 2005. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis* 8: 43–51.
62. Reya, T., A. W. Duncan, L. Ailles, J. Domen, D. C. Scherer, K. Willert, L. Hintz, R. Nusse, and I. L. Weissman. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 423: 409–414.
63. Staal, F. J. T., T. C. Luis, and M. M. Tiemessen. 2008. WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol* 8: 581–593.
64. Zhou, J., P. Cheng, J.-I. Youn, M. J. Cotter, and D. I. Gabrilovich. 2009. Notch and wingless signaling cooperate in regulation of dendritic cell differentiation. *Immunity* 30: 845–859.
65. Jiang, A., O. Bloom, S. Ono, W. Cui, J. Unternaehrer, S. Jiang, J. A. Whitney, J. Connolly, J. Banchereau, and I. Mellman. 2007. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27: 610–624.
66. Manicassamy, S., B. Reizis, R. Ravindran, H. Nakaya, R. M. Salazar-Gonzalez, Y. C. Wang, and B. Pulendran. 2010. Activation of β -Catenin in Dendritic Cells Regulates

Immunity Versus Tolerance in the Intestine. *Science* 329: 849–853.

67. Egan, C. E., S. B. Cohen, and E. Y. Denkers. 2012. Insights into inflammatory bowel disease using *Toxoplasma gondii* as an infectious trigger. *Immunol. Cell Biol.* 90: 668–675.

CHAPTER 2

Deployment of Mucosal Immune Defenses Against *Toxoplasma**

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Abstract

Toxoplasma gondii is a highly prevalent protozoan pathogen that is transmitted through oral ingestion of infectious cysts. As such, mucosal immune defenses in the intestine constitute the first and arguably most important line of resistance against the parasite. The response to infection is now understood to involve complex three-way interactions between *Toxoplasma*, the mucosal immune system and the host intestinal microbiota. Productive outcome of these interactions ensures resolution of infection in the intestinal mucosa. Nonsuccessful outcome may result in emergence of proinflammatory damage that can spell death for the host. Here, we discuss new advances in our understanding of the mechanisms underpinning these disparate outcomes, with particular reference to initiators, effectors and regulators of mucosal immunity stimulated by *Toxoplasma* in the intestine.

Introduction

The intestinal mucosa forms a dynamic arena in which principles of tolerance and immunity are dramatically played out throughout the host lifetime (1-3). Effective responses maintain homeostasis and protect from infection, but dysregulated immunity can lead to inability to control microbial incursion and may lead to chronic inflammatory disease in the gut mucosa and beyond (4-6).

The structure of the intestine sets the landscape within which these fundamentals of mucosal immunology are played out. The intestinal mucosa is composed of a single layer of epithelial cells with an embedded population of nonconventional intraepithelial lymphocytes. Underlying this, the lamina propria (LP) contains diverse cell types responsible for defense against invading pathogens, including T cells, macrophages and dendritic cells (DC), as well as those that maintain tolerance to commensal flora in the gut. Within the intestinal lumen itself, the vast number of commensals (on the order of 10^{14} in humans, with several thousand discrete species normally present) underscores the astounding ability to detect and respond to incoming pathogens while simultaneously avoiding inappropriate reactions to harmless or even beneficial occupants of the intestine. Studies largely in mice with the orally acquired protozoan parasite *Toxoplasma gondii* have served to illuminate these principles and have contributed key insights into inflammation and immunity in the intestinal mucosa during infection.

Toxoplasma is an exceedingly common parasite of humans and animals, and it is estimated that 10-50% of the human population are latently infected with the parasite worldwide. The vast majority of cases are asymptomatic, but the parasite may emerge as a life-threatening opportunistic pathogen in immunodeficient hosts and during congenital infection (7). Infection is acquired by ingestion of tissue cysts or oocysts shed by cats. Within the small intestine, *T. gondii* differentiates into the fast replicating and disseminating form known as the tachyzoite (8). Invasion of host cells by tachyzoites results in creation of a specialized parasitophorous vacuole within which *Toxoplasma* replicates, eventually leading to egress and entry into new host cells. Infection leads to spreading beyond the intestinal mucosa and culminates in cyst formation within skeletal muscle and central nervous system tissues. Within the latter tissue, toxoplasmic encephalitis may emerge as a life-threatening disease in immunosuppressed patients. In the feline intestine, the parasite undergoes sexual reproduction, resulting in fecal shedding of highly infectious oocysts into the environment. *T. gondii* is known for its ability to induce a strongly polarized Th1 response that is normally highly effective in host protection but that can also spiral out of control to cause host tissue damage and sometimes death (9-11). Although *Toxoplasma* spreads well beyond the borders of the intestinal mucosa, it is within this tissue that the first encounter between the parasite and host immune system occurs. As such, the mucosal immune response is key to determining the outcome of infection with this microbial pathogen.

Early mucosal targets of infection

In order to establish successful infection, *Toxoplasma* must breach the intestinal epithelial layer, after which it must disseminate and convert to the life-long latent form. Although tachyzoites may directly invade intestinal epithelial cells, in vitro studies employing polarized intestinal cell lines suggest the parasites use a more efficient transmigration mechanism dependent on a paracellular pathway. This pathway involves manipulation of host cell intercellular cell adhesion molecule (ICAM)-1 and occludin to pass through cellular tight junctions (12, 13). Interestingly, trans migratory capability seems to be genetically controlled in *Toxoplasma* and is linked to virulence of the parasite (14, 15).

Once through the epithelial barrier, *T. gondii* invades numerous cell types within the small intestinal LP. Parasites genetically engineered to express fluorescent markers were used to identify infected LP cells after infection, where monocytes and neutrophils were identified as the most frequently infected cells, followed by macrophages and lymphocytes, while dendritic cells (DC) comprised a low percentage of the infected population (16, 17). *T. gondii* forms discrete foci of replication within small intestinal villi, and two recent studies provide unexpected evidence that this occurs because the parasite travels in retrograde fashion through the intestinal lumen rather than the tissue to establish localized centers of infection. Interestingly, it also appears to be the case that infected LP neutrophils may trans migrate into the lumen to establish new infection foci (16, 17). A related role for retrograde neutrophil transmigration in the response to *Toxoplasma* comes from a recent study showing that

neutrophils emigrate into the intestinal lumen shortly after infection to form structures that encapsulate commensals, limiting their entry into the intestinal mucosa that would otherwise occur (18, 19).

Dendritic cells are well known for their capacity to take up soluble or particulate antigen and migrate to regional lymphoid organs. As such, they have also been proposed to be “Trojan horses” for parasite dissemination during oral and intraperitoneal infection (20, 21). Interestingly, DC infected with *T. gondii* acquire a hypermotility phenotype that is dependent upon parasite exploitation of GABAergic signaling in the host cell (22, 23). Adoptive transfer of infected DC results in faster parasite dissemination and increased parasite burden compared to transfer of free parasites (24). However, it has yet to be established that this phenomenon also operates in the intestinal mucosa. Regardless, while some studies indicate that LP DC are not a major infection reservoir, they may nevertheless contribute to dissemination of parasites from the mucosa into regional lymph nodes and beyond.

Role of mucosal dendritic cells in innate immune initiation

Concomitant with early invasion in the intestinal mucosa, it is essential for host resistance that IL-12 production is initiated to ensure production of protective IFN- γ by NK cells and newly generated Th1 T lymphocytes. Several studies have characterized cellular sources of IL-12 following oral *T. gondii* infection. While in vitro studies identify neutrophils, dendritic cells and inflammatory monocytes as cells that produce IL-12 in response to *T. gondii*, the relative role of these cells during in

vivo infection is less clear (25-28). While neutrophils may be an important in vivo source of IL-12 stimulating protective immunity, more current data indicate that neutrophil recruitment to the intestine contributes to small intestinal inflammatory pathology that is triggered by *Toxoplasma* (29, 30).

Dendritic cells are implicated as a primary source of IL-12 based upon i.p. and oral infection studies using the Cre-flox and diphtheria toxin systems, respectively, to delete cells expressing CD11c, a marker associated with DC (25, 31). In the case of oral infection, ablation of CD11c⁺ cells leads to elevated parasite burden and loss of the CD4⁺ T cell IFN- γ response. An important caveat in these studies is that CD11c can also be expressed by inflammatory monocytes, which themselves are observed to produce IL-12 during infection (29, 30, 32). Nevertheless, mice lacking Batf3, a transcription factor important for differentiation of CD8 α and tissue CD103 DC (33, 34), are defective in IL-12 production during *Toxoplasma* infection, providing compelling evidence for these specific DC subsets as sources of IL-12 during oral infection (35). Amongst these, CD103 DC may be the prime IL-12 source in the intestinal mucosa, as a previous study showed that intestinal CD8 α ⁻, but not CD8 α ⁺ DC, express IL-12 following oral infection (18).

A recent study employed diphtheria toxin to ablate cells expressing a newly identified transcription factor zDC (Zbtb46, Btbd4), which is exclusively expressed by conventional DC subsets and not by monocytes or plasmacytoid DC (pDC) (31). Mice that were ablated for these cells and then orally infected with *T. gondii* failed to reach

the same level of susceptibility and reduced Th1 activity as CD11c-depleted mice. This suggests that non-conventional DC or other cells that express CD11c, possibly including inflammatory monocytes, may also contribute to establishment of protective immunity to *T. gondii* (31). One scenario that seems particularly likely is that resident CD103⁺ DC in the intestine provide the initial burst of IL-12, and this is followed by activation and recruitment of inflammatory monocytes to the intestinal mucosa that might further contribute to the IL-12 response during infection, enabling optimal Th1 generation.

In addition to producing IL-12, mucosal DC have been shown to regulate T cell responses following *T. gondii* infection by production of the vitamin A metabolite retinoic acid (RA). Production of RA by mucosal DC promotes tolerogenic responses at steady-state by acting in concert with TGF- β to induce mucosal Treg (36-38). Because RA directly binds to T cells via RA receptor (RAR)- α and enhances T cell receptor signaling (39), more generalized effects of RA on T cells are possible. Indeed, loss of RAR- α signaling in the context of inflammation initiated by *Toxoplasma* infection results in defective T cell effector responses in the mucosal compartment and increased susceptibility to the parasite (39).

Immune recognition

How *T. gondii* is detected by the immune system and the role of Toll-like receptors (TLR) in this lynchpin event has been the subject of intense interest and investigation (40-42). Mice deficient in MyD88 and IRAK4, central mediators of TLR signaling,

are extremely susceptible to *Toxoplasma* infection (43-45). Furthermore, deficiency in UNC93B, a chaperone protein involved in trafficking intracellular TLR from the endoplasmic reticulum to the endosomal compartment, results in complete loss of resistance to *T. gondii* (46, 47). These results together point to intracellular TLR as being particularly important in immune detection of *Toxoplasma*.

The tachyzoite protein profilin (TgPRF) serves as an IL-12-inducing ligand for TLR11, which along with TLR3, 7, 8, 9 and 12, is expressed intracellularly (47, 48). More recently, it was found that TgPRF also activates immunity through binding to TLR12 (49-51). Current evidence indicates that TgPRF recognition by TLR11 or TLR12 homodimers or TLR11/12 heterodimers is a major event in innate immune recognition of *Toxoplasma*. Which particular TLR configuration takes primary importance may depend upon the cell type involved or the context of infection. While this is an area that has yet to be explored in detail, results in *Tlr11*^{-/-} mice indicate that signaling mediated by this receptor plays a role in proinflammatory Crohn's disease-like pathology in the intestine that is triggered by high dose infection (18).

Tachyzoite profilin is a molecule that is required for cell invasion (52). As a molecule essential for parasite survival, it fulfills a basic requirement for a pathogen-associated molecular pattern targeted for recognition by the immune system (53). The TgPRF protein is not actively secreted by *Toxoplasma*, and TLR11/12 do not observably traffic to the parasitophorous vacuole membrane. Therefore, it is most likely that

recognition at this level involves DC phagocytic uptake of dead parasites or debris, and indeed there is evidence that most IL-12 is produced by noninfected cells (54).

In addition to recognition of TgPRF by intracellular TLR, parasite nucleic acid recognition by TLR3, 7 and 9 also facilitates resistance to infection (49). Furthermore, TLR2 and TLR4 recognition of parasite glycosylphosphatidylinositol lipids at the cell surface may contribute to innate immune activation, although conclusions from these studies rely mostly on in vitro observations (55, 56). Regardless, the sum of these data strongly argues that several TLR, most prominently TLR11 and TLR12, act in concert to signal recognition of *T. gondii*. Yet, TLR11 and TLR12, while expressed in rodents, are absent in many species, including humans. In such cases, TgPRF is most likely less important, and other parasite molecules are likely to take the role of primary innate immunity triggers. It is possible that parasite nucleic acids and glycosylphosphatidylinositol lipids emerge as dominant TLR ligands in these cases.

The secretory dense granule protein GRA15, a molecule expressed in a parasite strain-dependent manner by low virulence *Toxoplasma*, has been shown to trigger NFkB activation and subsequent IL-12 production directly within infected cells (57). This is at odds with data indicating that *Toxoplasma* is recognized at a distance by noninfected cells, but some studies do in fact indicate that IL-12 is produced by infected cells (58). One recently proposed model that resolves the apparent discrepancy is that IL-12 at earliest points of infection may be produced by infected cells through GRA15, and later with the onset of fulminant infection, TgPRF uptake

and TLR stimulation in noninfected cells assumes the major source of IL-12 (59). Nevertheless, the strain-specificity of GRA15 expression would indicate that the cellular origins of IL-12 production must depend upon the infecting *Toxoplasma* isolate.

Luminal bacteria and *Toxoplasma* together control mucosal immunity during infection

Our understanding of innate immune recognition of *Toxoplasma* has outpaced similar knowledge of other eukaryotic microorganisms. Nevertheless, it has become clear that our understanding remains incomplete insofar as simultaneous recognition of luminal gut flora must also be considered. Commensal bacteria benefit the host by shaping mucosal immunity under steady-state conditions, but they also cause inflammation under disequilibrium situations. In the context of *Toxoplasma* in the intestine, the host microbiota plays similar roles.

Data pointing to the importance of the microbiota in oral *T. gondii* infection come from several lines of evidence. Following i.p. infection, TLR11 deficiency results in complete loss of DC-derived IL-12 and the subsequent IFN- γ response upon systemic *T. gondii* infection, and mice fail to control the infection, resulting in early mortality. Yet, these responses, while diminished, are nevertheless retained in the intestinal mucosa of *Tlr11*^{-/-} mice during mucosal infection, which is in direct contrast to infection in *MyD88*^{-/-} animals. Furthermore, antibiotic treatment of *Tlr11*^{-/-} animals prior to *T. gondii* infection abolishes the remaining IL-12 and Th1 responses and

increases susceptibility to oral infection, demonstrating the importance of gut bacteria in providing an immunostimulatory role in the absence of TgPRF recognition (18). Thus, the gut commensal population acts as an adjuvant facilitating development of an optimal protective immune response to *Toxoplasma*.

It was recently shown that oral infection with *T. gondii* directs an antigen-specific immune response not only against the parasite, but also against the microbiota itself. Through use of mice genetically engineered to express a T cell receptor specific for commensal-derived flagellin, Hand et al. showed that a population of CD4⁺ T cells induced by *T. gondii* infection respond directly to bacterial flagellin through proliferation and Th1 differentiation. Surprisingly, the commensal-specific T cell population is comparable to that of the parasite-specific cells both in size and behavior. Furthermore, these flagellin-specific cells develop into functional memory cells (60). *T. gondii* infection in the gut therefore seems to trigger loss of tolerance to intestinal bacteria, possibly due to infection-induced epithelial damage and consequent bacterial translocation. In turn, this results in T cell priming against bacterial antigens, but the bacteria also provide a source of PAMPs that facilitate ignition of the protective Th1 response against *Toxoplasma* itself (Figure 2.1).

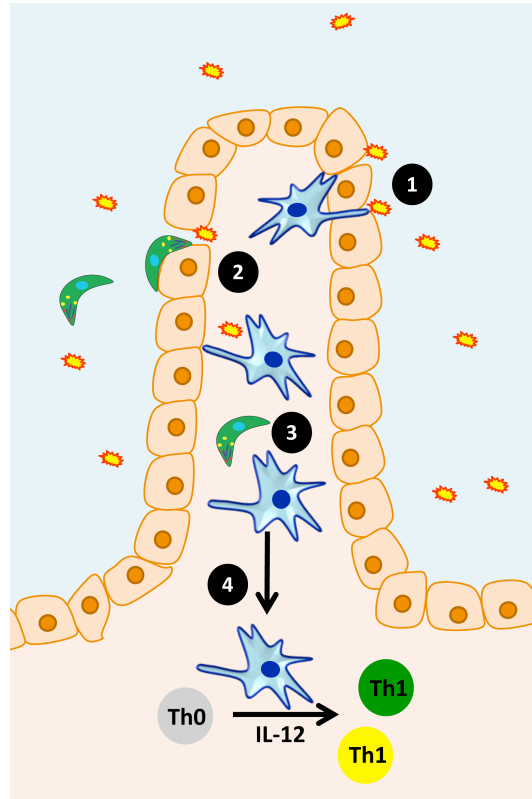


Figure 2.1. Gut commensal bacteria serve as adjuvants and antigens for *Toxoplasma* triggered IL-12 and Th1 induction. (1) Gut resident CX3CR1⁺ macrophages and dendritic cells (DC) continually sample bacterial antigens from the intestinal lumen by extending dendrites between epithelial cells. (2) Mucosal DC may also sample bacteria that have translocated to the LP as a result of *Toxoplasma*-mediated disruption of the epithelial barrier associated with infection. This may occur during parasite paracellular migration or by direct infection and lysis of epithelial cells. (3) DC acquire *Toxoplasma* antigen either by direct infection or by uptake of parasite material. (4) Activated antigen-bearing DC migrate to the draining lymph node and produce IL-12, resulting in the activation of Th1 cell populations specific for commensals and *T. gondii* itself.

It is also clear that gut bacteria can exacerbate *T. gondii* infection in a manner that is similar to that thought to occur in inflammatory bowel disease (IBD) pathogenesis (5). High dose oral infection with *T. gondii* leads to an IBD-like phenotype in C57BL/6 mice, which is associated with CD4⁺ T cell infiltration and overexpression of proinflammatory cytokines, including IFN- γ , IL-12 and TNF- α , as well as IL-22 and possibly IL-17 (although this is controversial) (61-66). Additionally, *T. gondii* alters the composition of the gut microbiota, resulting in a global reduction in bacterial diversity, a switch from Gram-positive to Gram-negative bacteria and an expansion of adherent and invasive pathobionts that results in translocation into the LP (67, 68).

It is clear that imbalances in the intestinal microbiota are a cause of inflammation during *T. gondii* infection. For example, administration of antibiotics prior to *Toxoplasma* infection protects mice from parasite-induced ileitis, and gnotobiotic mice are resistant to parasite-induced ileitis, confirming the role of the microbiota in promoting intestinal lesions upon *T. gondii* infection (67). Mice deficient for TLR4 display decreased IFN- γ and NO levels in the small intestine and are more resistant to infection, most likely resulting from lost recognition of bacterial TLR4 ligands (68). Furthermore, antibiotic treatment of recipient mice protects against ileitis induced by adoptive transfer of pathogenic LP CD4⁺ T cells and IEL from *Toxoplasma*-infected animals (69). These collective studies emphasize the dual potential role of luminal microbiota in stimulating protective immunity with low dose parasite inocula versus switching to driving an inflammatory cytokine storm that surrounds high dose infection.

The precise trigger provided by *T. gondii* that results in loss of tolerance to gut flora and intestinal dysbiosis has remained enigmatic, but recent studies suggest that parasite effects on Paneth cells may be the key to pathogenesis. Paneth cells are specialized epithelial cells located in intestinal crypts, and they secrete antimicrobial peptides such as defensins into the intestinal lumen (70). One study found that, early after oral infection, Paneth cells degranulate in a TLR9-dependent manner, resulting in the release of antimicrobial Cationic Peptides that somehow promote optimal Th1 responses to the parasite (71). More directly related to the onset of inflammation, it was recently found that *Toxoplasma* infection causes loss of Paneth cells, an event that in turn leads to dysbiosis and fulminant pathology (72). Interestingly, germ-free mice infected with *T. gondii* retain Paneth cells, although they are again eliminated upon the addition of *Enterobacteriaceae*, but not *Bacteroides*. This suggests that Paneth cell loss is dependent on the combined effect of the parasite and Proteobacteria that emerge as dominant species during intestinal inflammation. Loss of Paneth cells is dependent on CD4⁺ T cell IFN- γ production via T cell-intrinsic MyD88 signaling, as Lck-Cre *Myd88^{fl/fl}* mice phenocopy *Myd88^{-/-}* animals, which display unaltered Paneth cells and antimicrobial peptide expression (72). Thus, this study provides a model to understand how *Toxoplasma* triggers inflammation-mediated dysbiosis in the intestine. Early IL-12-dependent induction of Th1 cells specific for *Toxoplasma* and intestinal flora causes IFN- γ -dependent Paneth cell depletion. In turn, loss of a major source of antimicrobial peptides in the small intestine results in overgrowth of pathobionts associated with dysbiosis and associated inflammatory lesions (Figure 2.2). This has

particular relevance for understanding ileal Crohn's pathogenesis because independent studies have implicated dysfunctional Paneth cell responses as underlying onset of disease(73).

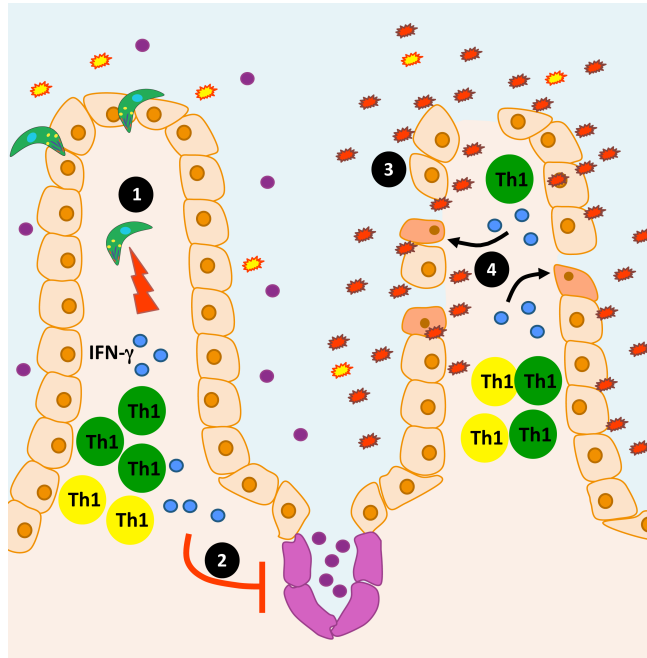


Figure 2.2. Control of infection and emergence of dysbiosis during *Toxoplasma* infection. (1) IFN- γ produced by Th1 lymphocytes and NK cells (not shown) induces killing of parasites and resolution of infection. The degree to which parasite- and commensal-specific T cells (denoted in yellow and green) contribute to killing is presently unclear. (2) During emergence of dysbiosis, infection-induced IFN- γ promotes Paneth cell elimination. Normally, these cells secrete antimicrobial peptides (AMP) into the intestinal lumen to maintain homeostatic levels of commensal populations, including the healthy Gram-positive *Bacteroides* (shown in yellow). (3) Expansion of pathogenic Gram-negative *Enterobacteriaceae* (shown in red), which adhere to the epithelium, cause barrier damage and invade the intestinal mucosa, is caused by loss of Paneth cell-dependent AMP. (4) Damage may be further exacerbated by the continued proinflammatory activity of Th1 effectors responding to bacteria and parasite antigen.

Role of inflammatory monocytes in the intestinal mucosa

While inflammatory monocytes may be a significant IL-12 source during *T. gondii* infection, their more important function is most likely as chemokine receptor CCR2-dependent microbicidal effector cells (74). Pioneering studies demonstrated the importance of similar cells in defense against *Listeria monocytogenes* in the spleen (75). Subsequently, CCR2-dependent recruitment of these cells into the intestinal mucosa of *Toxoplasma* infected mice was found to control parasite replication (32). Rather than controlling migration to the inflamed intestinal LP, CCR2 appears necessary for exit from the bone marrow (76). The recruitment of inflammatory monocytes into the intestine now appears to be mediated by a CCL3/CCR1 axis (77), a finding that confirms older studies showing a requirement for CCR1 in control of infection (78). Thus, LP IL-15-dependent NKp46⁺ innate lymphoid cells activated by infection-induced IL-18 produce CCL3 that in turn chemoattracts CCR1-positive inflammatory monocytes to the intestinal mucosa (Figure 2.3).

Inflammatory monocytes must be activated by IFN- γ to acquire effector function. In an i.p. infection model, NK cells may provide this critical source (79), but our recent studies further identified Th1 T cells, recruited to the site of infection in dependence upon expression of CXCR3, as an important source of this cytokine in this capacity (29). Thus, we can define an intricate chemokine network surrounding *Toxoplasma* in the intestine, in which CCR1/CCL3 recruits CCR2-dependent monocytes, while approximately simultaneously, Th1 effector cells are recruited

through CXCR3 to effect optimal inflammatory monocyte activation for parasite control (Figure 2.3).

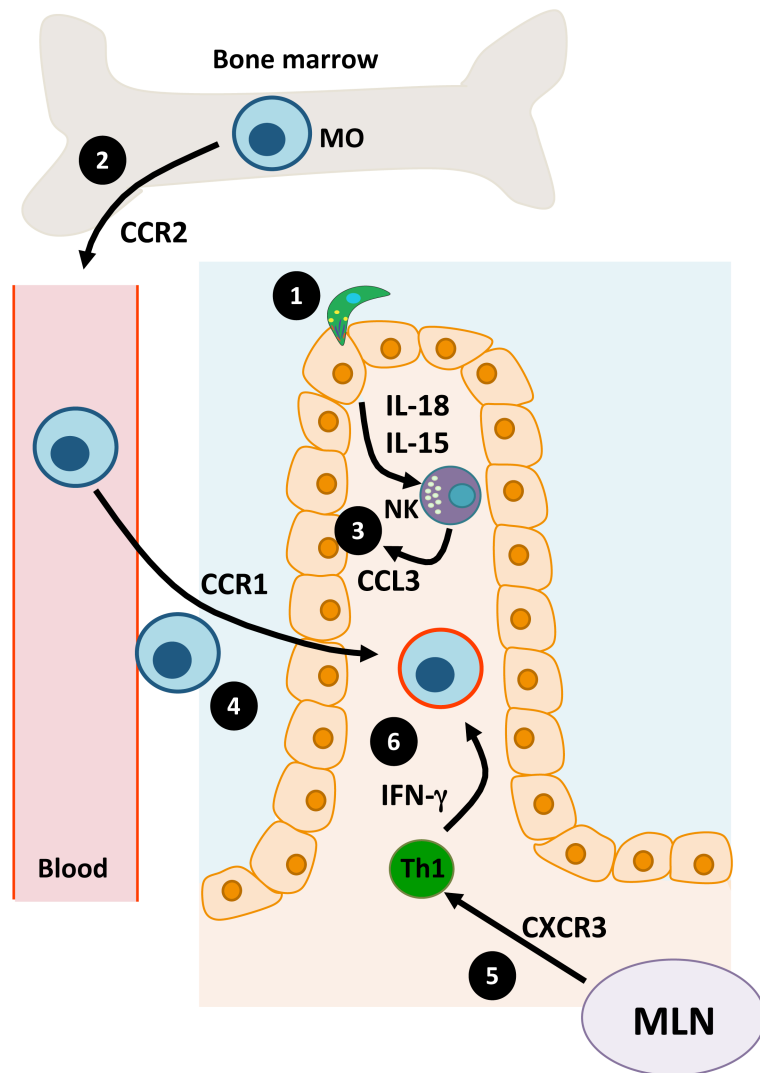


Figure 2.3. Chemokine network surrounding inflammatory monocyte recruitment and activation during *T. gondii* infection. (1) Upon intestinal infection, non-hematopoietic cells, such as epithelial cells, are induced to secrete cytokines IL-18 and IL-15, resulting in activation and maturation of NKp46⁺ innate-like lymphocytes. (2) Concurrently, monocytes are recruited from the bone marrow to the bloodstream in dependence upon CCR2. (3) In the intestinal LP, activated, innate-like lymphocytes secrete chemokines, such as CCL3. (4) Recruitment of monocytes expressing CCR1, a receptor for CCL3, promotes accumulation in the intestinal LP. (5) Local production of IFN- γ -inducible chemokines following *T. gondii* infection recruits CXCR3-expressing Th1 cells presumably from the mesenteric lymph node (MLN). (6) High level secretion of IFN- γ by CXCR3-positive CD4 T cells activates LP inflammatory monocytes, resulting in upregulated antimicrobial effector function and enabling control of the parasite.

It is not yet clear how inflammatory monocytes within the intestine kill parasites. The cells were first defined based in part on their ability to express iNOS and produce the microbicidal molecule nitric oxide (74, 80). However, because iNOS-deficient mice survive acute infection (in contrast to *Ifn γ ^{-/-}* mice) (81, 82), this killing mechanism is unlikely. Instead, it is more likely that immunity-related GTPase (IRG) proteins are key to parasite killing. IRG effector molecules are strongly induced by IFN- γ , and recent data show they accumulate on the parasitophorous vacuole membrane to mediate its destruction, thereby depriving the parasite of its essential intracellular niche (83-86). Nevertheless, IRG studies have mostly employed in vitro infection systems, and while some IRG knockout mice are indeed increased in susceptibility during oral infection (87), formal data demonstrating a requirement for this activity in mucosal inflammatory monocyte killing is lacking. There is also emerging evidence that members of the p65 guanylate-binding protein (GBP) family play a role in parasite killing, as deletion of 6 GBP genes resulted in defective recruitment of *Irgb6* to IFN- γ stimulated macrophages in vitro as well as enhanced parasite replication in vitro and in vivo, although a role for these proteins during oral infection has yet to be confirmed (88).

A more complex view of inflammatory monocytes comes from a recent study indicating immunoregulatory properties in the response to *Toxoplasma* (89). Thus, these cells acquire a tissue-specific regulatory phenotype that is dependent upon commensals in the gut. This property is associated with production of the lipid mediator prostaglandin E2 (PGE2), a molecule known to have immunoregulatory

functions in the intestinal mucosa (90). In turn, PGE₂ inhibits activation of neutrophils that also accumulate in the intestinal LP during *T. gondii* infection. In the absence of CCR2-dependent inflammatory monocyte recruitment, neutrophil activation and concomitant release of factors, such as TNF- α and reactive oxygen species, results in emergence of intestinal lesions. Thus, inflammatory monocytes recruited to the intestine possess the dual properties of controlling parasite growth and down-modulating PMN-mediated intestinal pathology.

Intraepithelial lymphocyte function during *Toxoplasma* infection

Intraepithelial lymphocytes (IEL) constitute a complex population of T cells embedded within the intestinal epithelium. They are normally present at approximately one per 10 epithelial cells, although this can vary considerably depending upon inflammation and infection. In mice, the majority of IEL express either CD8 $\alpha\beta$ or CD8 $\alpha\alpha$ and, amongst the latter, a large proportion expresses the $\gamma\delta$ rather than $\alpha\beta$ T cell receptor (TCR). The origin, antigen specificity, MHC restriction and function of these cells are complex and not well understood (91). Nevertheless, growing evidence suggests that under normal conditions these cells serve as important regulators of homeostasis, and dysregulation in the IEL compartment contributes to pathology associated with human IBD (92).

During *T. gondii* infection, intestinal epithelial tight junction proteins are abnormally affected and barrier function is compromised, resulting in decreased control of parasite spread (93). Others have shown that CD8 $\alpha\beta$ IEL isolated from *T. gondii*-infected mice

produce IFN- γ and are capable of cytolytic function against infected enterocytes in vitro (94). When adoptively transferred, these IEL traffic to the small intestinal mucosa in dependence upon CCR5 and provide protection against challenge infection (95-97). Perhaps most interestingly, IFN- γ is required in recipient animals but not donor IEL themselves (98).

Populations within the IEL compartment also functionally interact with CD4⁺ T cells in the LP. For example, CD4⁺ T lymphocytes somehow interact with infected epithelial cells to promote proinflammatory cytokine production (99). CD8ab IEL down-regulate this activity in dependence upon TGF- β production, suggesting that they play a role in protecting against inflammation in the intestine (95, 100). Nevertheless, within the IEL population as a whole is embedded the potential to contribute to inflammatory pathology during infection (101). During high dose infection in C57BL/6 mice, $\alpha\beta$ TCR CD8 α IEL mediate damage that is dependent upon their expression of CCR2 (102). The pathogenic IEL population that emerges under inflammatory conditions does not function alone, but instead requires LP-derived CD4 T cells for fulminant pathology (69). In this situation, IEL play a role in recruiting CD4⁺ T lymphocytes into the intraepithelial compartment, providing a mechanistic scenario for the original observation that CD4⁺ T cells are required for parasite-mediated ileal damage (103). Taken together, these data indicate that under low dose inocula, IEL seem to be important immunoregulatory cells that prevent onset of intestinal pathology and may even contribute to control of infection. However, under high dose conditions, these cells switch to a dysregulated proinflammatory

phenotype that contributes to lesion development in the small intestine. Whether this reflects actual switching in the function of a single IEL population or instead results from the changing dominance of one IEL population over another is not at present understood. Furthermore, while the gut microbiota is required for the pathogenic effects of IEL, little is known about how the intestinal flora interact and drive the function of these cells.

Toxoplasma controls the fate of intestinal Treg

Within the intestinal mucosa resides a large population of FoxP3⁺ regulatory T cells (Treg) composed of steady-state cells (nTreg) and cells induced by infection or inflammation (iTreg). These cells are well known to be important in quelling inflammation and maintaining tolerance in the intestine, in large part through production of anti-inflammatory IL-10 (104). During *Toxoplasma* infection, IL-10 clearly has an important role since *Il10*^{-/-} mice rapidly succumb due to proinflammatory cytokine overproduction (105). Within the context of oral infection, susceptibility due to IL-10 loss is associated with emergence of Crohn's like pathology in the small intestine (106).

During lethal oral infection, Foxp3⁺ Treg in the intestine express IL-10 under the direction of local IL-27 signaling and have the capacity to suppress T cell responses (107), although interestingly they co-express IFN- γ and T-bet. Nevertheless, the intestinal Treg population collapses in size with the emergence of inflammatory pathology (108). This was shown to be due to expansion of pathogen-specific T cells

with limited IL-2 producing capacity, leading to the cytokine starvation of Treg (109). Thus, reversal of this collapse was accomplished by the addition of recombinant IL-2/anti-IL-2 complexes, resulting in fewer effector T cell populations and ameliorated pathology. Importantly, the collapse in the Treg population is not restricted to a lethal *Toxoplasma* dose, as a similar loss in Treg occurs under low dose non-lethal parasite infection (our unpublished observations and Benson *et al.*) (109). Indeed, it is tempting to speculate that emergence of microbiota-specific T cells associated with *Toxoplasma* infection is enabled by extinguishing tolerogenic Treg in the intestine.

If Treg disappearance in the intestine is a characteristic of *Toxoplasma* infection, what is the source of IL-10 that protects against immune-mediated disease? First, it is possible that sufficient numbers of Treg are maintained during nonlethal infection to provide protective IL-10. Second, it is possible that other cell types provide IL-10. The finding that CD4⁺ T cell-derived IL-10 is necessary to prevent immunopathology during oral *T. gondii* infection does not distinguish between conventional CD4⁺ T lymphocytes and Treg cells (110). However, in an i.p. infection model it was indeed found that IFN- γ -producing Th1 cells switch to IL-10 production as infection progresses (111). Other studies have implicated natural killer cells as well as NKT cells as significant IL-10 sources during *Toxoplasma* infection (112, 113). Clearly, more work is required to resolve these issues.

Conclusions and future directions

Our knowledge of the mucosal immune responses that are galvanized into action by *Toxoplasma* and other microbial pathogens has expanded greatly in recent years. Yet, there is still much that remains unknown. We do not fully understand how cells of the mucosal immune system successfully integrate signals received simultaneously from microbial pathogens and commensal microbiota during infection, nor do we understand the breakdown in tolerance that leads to dysbiosis in the intestine. Knowledge gained through use of experimental models such as *Toxoplasma* will lead to new ways of understanding as well as treating infection and inflammation at the borders of the intestinal mucosa.

References

1. Kayama, H., and K. Takeda. 2012. Regulation of intestinal homeostasis by innate and adaptive immunity. *Int. Immunol.* 24: 673–680.
2. Nutsch, K. M., and C.-S. Hsieh. 2012. T cell tolerance and immunity to commensal bacteria. *Curr. Opin. Immunol.* 24: 385–391.
3. Sansonetti, P. J. 2011. To be or not to be a pathogen: that is the mucosally relevant question. *Mucosal Immunol* 4: 8–14.
4. Cader, M. Z., and A. Kaser. 2013. Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation. *Gut* 62: 1653–1664.
5. Kaser, A., S. Zeissig, and R. S. Blumberg. 2010. Inflammatory bowel disease. *Annu. Rev. Immunol.* 28: 573–621.
6. Xavier, R. J., and D. K. Podolsky. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448: 427–434.
7. McLeod, R., C. Van Tubbergen, J. G. Montoya, and E. Petersen. 2014. Human *Toxoplasma* Infection. In *Toxoplasma gondii*, 2nd ed. L. M. Weiss, and K. Kim, eds. Academic Press. 100–162.
8. Dubey, J. P. 2014. The History and Life Cycle of *Toxoplasma gondii*. In *Toxoplasma gondii*, 2nd ed. L. M. Weiss, and K. Kim, eds. Academic Press. 1–20.
9. Denkers, E. Y., and R. T. Gazzinelli. 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin. Microbiol. Rev.* 11: 569–588.
10. Dupont, C. D., D. A. Christian, and C. A. Hunter. 2012. Immune response and immunopathology during toxoplasmosis. *Semin Immunopathol* 34: 793–813.
11. Egan, C. E., S. B. Cohen, and E. Y. Denkers. Insights into inflammatory bowel disease using *Toxoplasma gondii* as an infectious trigger. *Immunol. Cell Biol.* 90: 668–675.
12. Barragan, A., F. Brossier, and L. D. Sibley. 2005. Transepithelial migration of *Toxoplasma gondii* involves an interaction of intercellular adhesion molecule 1 (ICAM-1) with the parasite adhesin MIC2. *Cell. Microbiol.* 7: 561–568.
13. Weight, C. M., and S. R. Carding. 2012. The protozoan pathogen *Toxoplasma gondii* targets the paracellular pathway to invade the intestinal epithelium. *Ann. N. Y. Acad. Sci.* 1258: 135–142.

14. Barragan, A., and L. D. Sibley. 2002. Transepithelial migration of *Toxoplasma gondii* is linked to parasite motility and virulence. *J. Exp. Med.* 195: 1625–1633.
15. Barragan, A., and L. D. Sibley. 2003. Migration of *Toxoplasma gondii* across biological barriers. *Trends Microbiol.* 11: 426–430.
16. Gregg, B., B. C. Taylor, B. John, E. D. Tait Wojno, N. M. Girgis, N. Miller, S. Wagage, D. S. Roos, and C. A. Hunter. 2013. Replication and distribution of *Toxoplasma gondii* in the small intestine after oral infection with tissue cysts. *Infection and Immunity* 81: 1635–1643.
17. Coombes, J. L., B. A. Charsar, S.-J. Han, J. Halkias, S. W. Chan, A. A. Koshy, B. Striepen, and E. A. Robey. 2013. Motile invaded neutrophils in the small intestine of *Toxoplasma gondii*-infected mice reveal a potential mechanism for parasite spread. *Proc. Natl. Acad. Sci. U.S.A.* 110: E1913–22.
18. Benson, A., R. Pifer, C. L. Behrendt, L. V. Hooper, and F. Yarovinsky. 2009. Gut commensal bacteria direct a protective immune response against *Toxoplasma gondii*. *Cell Host Microbe* 6: 187–196.
19. Molloy, M. J., J. R. Grainger, N. Bouladoux, T. W. Hand, L. Y. Koo, S. Naik, M. Quinones, A. K. Dzutsev, J.-L. Gao, G. Trinchieri, P. M. Murphy, and Y. Belkaid. 2013. Intraluminal containment of commensal outgrowth in the gut during infection-induced dysbiosis. *Cell Host Microbe* 14: 318–328.
20. Bierly, A. L., W. J. Shufesky, W. Sukhumavasi, A. E. Morelli, and E. Y. Denkers. 2008. Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during *Toxoplasma gondii* infection. *The Journal of Immunology* 181: 8485–8491.
21. Courret, N., S. Darche, P. Sonigo, G. Milon, D. Buzoni-Gatel, and I. Tardieux. 2006. CD11c- and CD11b-expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain. *Blood* 107: 309–316.
22. Fuks, J. M., R. B. G. Arrighi, J. M. Weidner, S. Kumar Mendu, Z. Jin, R. P. A. Wallin, B. Rethi, B. Birnir, and A. Barragan. 2012. GABAergic signaling is linked to a hypermigratory phenotype in dendritic cells infected by *Toxoplasma gondii*. *PLoS Pathog* 8: e1003051.
23. Weidner, J. M., and A. Barragan. 2013. Tightly regulated migratory subversion of immune cells promotes the dissemination of *Toxoplasma gondii*. *Int. J. Parasitol.*
24. Lambert, H., N. Hitziger, I. Dellacasa, M. Svensson, and A. Barragan. 2006. Induction of dendritic cell migration upon *Toxoplasma gondii* infection potentiates parasite dissemination. *Cell. Microbiol.* 8: 1611–1623.

25. Liu, C.-H., Y.-T. Fan, A. Dias, L. Esper, R. A. Corn, A. Bafica, F. S. Machado, and J. Aliberti. 2006. Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against *Toxoplasma gondii* infection in mice. *J. Immunol.* 177: 31–35.
26. Bliss, S. K., Y. Zhang, and E. Y. Denkers. 1999. Murine neutrophil stimulation by *Toxoplasma gondii* antigen drives high level production of IFN-gamma-independent IL-12. *J. Immunol.* 163: 2081–2088.
27. Hou, B., A. Benson, L. Kuzmich, A. L. DeFranco, and F. Yarovinsky. 2011. Critical coordination of innate immune defense against *Toxoplasma gondii* by dendritic cells responding via their Toll-like receptors. *Proc. Natl. Acad. Sci. U.S.A.* 108: 278–283.
28. Pepper, M., F. Dzierzinski, E. Wilson, E. Tait, Q. Fang, F. Yarovinsky, T. M. Laufer, D. Roos, and C. A. Hunter. 2008. Plasmacytoid dendritic cells are activated by *Toxoplasma gondii* to present antigen and produce cytokines. *J. Immunol.* 180: 6229–6236.
29. Cohen, S. B., K. J. Maurer, C. E. Egan, S. Oghumu, A. R. Satoskar, and E. Y. Denkers. 2013. CXCR3-dependent CD4⁺ T cells are required to activate inflammatory monocytes for defense against intestinal infection. *PLoS Pathog* 9: e1003706.
30. Dunay, I. R., A. Fuchs, and L. D. Sibley. 2010. Inflammatory monocytes but not neutrophils are necessary to control infection with *Toxoplasma gondii* in mice. *Infection and Immunity* 78: 1564–1570.
31. Meredith, M. M., K. Liu, G. Darrasse-Jeze, A. O. Kamphorst, H. A. Schreiber, P. Guernonprez, J. Idoyaga, C. Cheong, K.-H. Yao, R. E. Niec, and M. C. Nussenzweig. 2012. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *Journal of Experimental Medicine* 209: 1153–1165.
32. Dunay, I. R., R. A. Damatta, B. Fux, R. Presti, S. Greco, M. Colonna, and L. D. Sibley. 2008. Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. *Immunity* 29: 306–317.
33. Edelson, B. T., W. KC, R. Juang, M. Kohyama, L. A. Benoit, P. A. Klekotka, C. Moon, J. C. Albring, W. Ise, D. G. Michael, D. Bhattacharya, T. S. Stappenbeck, M. J. Holtzman, S.-S. J. Sung, T. L. Murphy, K. Hildner, and K. M. Murphy. 2010. Peripheral CD103⁺ dendritic cells form a unified subset developmentally related to CD8alpha⁺ conventional dendritic cells. *Journal of Experimental Medicine* 207: 823–836.
34. Jaiswal, H., M. Kaushik, R. Sougrat, M. Gupta, A. Dey, R. Verma, K. Ozato, and P. Tailor. 2013. Batf3 and Id2 Have a Synergistic Effect on Irf8-Directed Classical

CD8 α ⁺ Dendritic Cell Development. *The Journal of Immunology* 191: 5993–6001.

35. Mashayekhi, M., M. M. Sandau, I. R. Dunay, E. M. Frickel, A. Khan, R. S. Goldszmid, A. Sher, H. L. Ploegh, T. L. Murphy, L. D. Sibley, and K. M. Murphy. 2011. CD8 α (⁺) dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites. *Immunity* 35: 249–259.

36. Mucida, D., Y. Park, G. Kim, O. Turovskaya, I. Scott, M. Kronenberg, and H. Cheroutre. 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317: 256–260.

37. Coombes, J. L., K. R. R. Siddiqui, C. V. Arancibia-Cárcamo, J. Hall, C.-M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103⁺ DCs induces Foxp3⁺ regulatory T cells via a TGF- β and retinoic acid-dependent mechanism. *J. Exp. Med.* 204: 1757–1764.

38. Sun, C.-M., J. A. Hall, R. B. Blank, N. Bouladoux, M. Oukka, J. R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J. Exp. Med.* 204: 1775–1785.

39. Hall, J. A., J. L. Cannons, J. R. Grainger, L. M. Dos Santos, T. W. Hand, S. Naik, E. A. Wohlfert, D. B. Chou, G. Oldenhove, M. Robinson, M. E. Grigg, R. Kastenmayer, P. L. Schwartzberg, and Y. Belkaid. 2011. Essential role for retinoic acid in the promotion of CD4(⁺) T cell effector responses via retinoic acid receptor alpha. *Immunity* 34: 435–447.

40. Denkers, E. Y. 2010. Toll-Like Receptor Initiated Host Defense against *Toxoplasma gondii*. *J. Biomed. Biotech.* 2010: 1–8.

41. Gazzinelli, R. T., and E. Y. Denkers. 2006. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol* 6: 895–906.

42. Yarovinsky, F. 2008. Toll-like receptors and their role in host resistance to *Toxoplasma gondii*. *Immunol. Lett.* 119: 17–21.

43. Béla, S. R., M. S. Dutra, E. Mui, A. Montpetit, F. S. Oliveira, S. C. Oliveira, R. M. E. Arantes, L. R. Antonelli, R. McLeod, and R. T. Gazzinelli. 2012. Impaired innate immunity in mice deficient in interleukin-1 receptor-associated kinase 4 leads to defective type 1 T cell responses, B cell expansion, and enhanced susceptibility to infection with *Toxoplasma gondii*. *Infection and Immunity* 80: 4298–4308.

44. Scanga, C. A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E. Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by

dendritic cells. *J. Immunol.* 168: 5997–6001.

45. Sukhumavasi, W., C. E. Egan, A. L. Warren, G. A. Taylor, B. A. Fox, D. J. Bzik, and E. Y. Denkers. 2008. TLR adaptor MyD88 is essential for pathogen control during oral toxoplasma gondii infection but not adaptive immunity induced by a vaccine strain of the parasite. *The Journal of Immunology* 181: 3464–3473.

46. Melo, M. B., P. Kasperkovitz, A. Cerny, S. Könen-Waisman, E. A. Kurt-Jones, E. Lien, B. Beutler, J. C. Howard, D. T. Golenbock, and R. T. Gazzinelli. 2010. UNC93B1 mediates host resistance to infection with *Toxoplasma gondii*. *PLoS Pathog* 6: e1001071.

47. Pifer, R., A. Benson, C. R. Sturge, and F. Yarovinsky. 2011. UNC93B1 is essential for TLR11 activation and IL-12-dependent host resistance to *Toxoplasma gondii*. *J. Biol. Chem.* 286: 3307–3314.

48. Yarovinsky, F., D. Zhang, J. F. Andersen, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh, and A. Sher. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 308: 1626–1629.

49. Andrade, W. A., M. D. C. Souza, E. Ramos-Martinez, K. Nagpal, M. S. Dutra, M. B. Melo, D. C. Bartholomeu, S. Ghosh, D. T. Golenbock, and R. T. Gazzinelli. 2013. Combined action of nucleic acid-sensing Toll-like receptors and TLR11/TLR12 heterodimers imparts resistance to *Toxoplasma gondii* in mice. *Cell Host Microbe* 13: 42–53.

50. Koblansky, A. A., D. Jankovic, H. Oh, S. Hieny, W. Sungnak, R. Mathur, M. S. Hayden, S. Akira, A. Sher, and S. Ghosh. 2012. Recognition of Profilin by Toll-like Receptor 12 Is Critical for Host Resistance to *Toxoplasma gondii*. *Immunity*.

51. Raetz, M., A. Kibardin, C. R. Sturge, R. Pifer, H. Li, E. Burstein, K. Ozato, S. Larin, and F. Yarovinsky. 2013. Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to *Toxoplasma gondii* profilin. *The Journal of Immunology* 191: 4818–4827.

52. Plattner, F., F. Yarovinsky, S. Romero, D. Didry, M.-F. Carlier, A. Sher, and D. Soldati-Favre. 2008. *Toxoplasma* profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. *Cell Host Microbe* 3: 77–87.

53. Janeway, C. A. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1: 1–13.

54. Pifer, R., and F. Yarovinsky. 2011. Innate responses to *Toxoplasma gondii* in mice

and humans. *Trends Parasitol* 27: 388–393.

55. Debierre-Grockiego, F., M. A. Campos, N. Azzouz, J. Schmidt, U. Bieker, M. G. Resende, D. S. Mansur, R. Weingart, R. R. Schmidt, D. T. Golenbock, R. T. Gazzinelli, and R. T. Schwarz. 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from *Toxoplasma gondii*. *J. Immunol.* 179: 1129–1137.

56. Debierre-Grockiego, F., N. Molitor, R. T. Schwarz, and C. G. K. Lüder. 2009. *Toxoplasma gondii* glycosylphosphatidylinositols up-regulate major histocompatibility complex (MHC) molecule expression on primary murine macrophages. *Innate Immun* 15: 25–32.

57. Rosowski, E. E., D. Lu, L. Julien, L. Rodda, R. A. Gaiser, K. D. C. Jensen, and J. P. J. Saeij. 2011. Strain-specific activation of the NF-kappaB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. *Journal of Experimental Medicine* 208: 195–212.

58. Kim, L., B. A. Butcher, C. W. Lee, S. Uematsu, S. Akira, and E. Y. Denkers. 2006. *Toxoplasma gondii* genotype determines MyD88-dependent signaling in infected macrophages. *J. Immunol.* 177: 2584–2591.

59. Melo, M. B., K. D. C. Jensen, and J. P. J. Saeij. 2011. *Toxoplasma gondii* effectors are master regulators of the inflammatory response. *Trends Parasitol* 27: 487–495.

60. Hand, T. W., L. M. Dos Santos, N. Bouladoux, M. J. Molloy, A. J. Pagán, M. Pepper, C. L. Maynard, C. O. Elson, and Y. Belkaid. 2012. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. *Science* 337: 1553–1556.

61. Guiton, R., V. Vasseur, S. Charron, M. T. Arias, N. Van Langendonck, D. Buzoni-Gatel, B. Ryffel, and I. Dimier-Poisson. 2010. Interleukin 17 receptor signaling is deleterious during *Toxoplasma gondii* infection in susceptible BL6 mice. *J. Infect. Dis.* 202: 427–435.

62. Kelly, M. N., J. K. Kolls, K. Happel, J. D. Schwartzman, P. Schwarzenberger, C. Combe, M. Moretto, and I. A. Khan. 2005. Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against *Toxoplasma gondii* infection. *Infection and Immunity* 73: 617–621.

63. Muñoz, M., M. M. Heimesaat, K. Danker, D. Struck, U. Lohmann, R. Plickert, S. Bereswill, A. Fischer, I. R. Dunay, K. Wolk, C. Loddenkemper, H.-W. Krell, C. Libert, L. R. Lund, O. Frey, C. Hölscher, Y. Iwakura, N. Ghilardi, W. Ouyang, T. Kamradt, R. Sabat, and O. Liesenfeld. 2009. Interleukin (IL)-23 mediates *Toxoplasma gondii*-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22

but independent of IL-17. *Journal of Experimental Medicine* 206: 3047–3059.

64. Craven, M., C. E. Egan, S. E. Dowd, S. P. McDonough, B. Dogan, E. Y. Denkers, D. Bowman, E. J. Scherl, and K. W. Simpson. 2012. Inflammation drives dysbiosis and bacterial invasion in murine models of ileal Crohn's disease. *PLoS ONE* 7: e41594.

65. Liesenfeld, O., H. Kang, D. Park, T. A. Nguyen, C. V. Parkhe, H. Watanabe, T. Abo, A. Sher, J. S. Remington, and Y. Suzuki. 1999. TNF-alpha, nitric oxide and IFN-gamma are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible mice infected perorally with *Toxoplasma gondii*. *Parasite Immunol.* 21: 365–376.

66. Liesenfeld, O. 2002. Oral infection of C57BL/6 mice with *Toxoplasma gondii*: a new model of inflammatory bowel disease? *J. Infect. Dis.* 185 Suppl 1: S96–101.

67. Heimesaat, M. M., S. Bereswill, A. Fischer, D. Fuchs, D. Struck, J. Niebergall, H.-K. Jahn, I. R. Dunay, A. Moter, D. M. Gescher, R. R. Schumann, U. B. Göbel, and O. Liesenfeld. 2006. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. *J. Immunol.* 177: 8785–8795.

68. Heimesaat, M. M., A. Fischer, H.-K. Jahn, J. Niebergall, M. Freudenberg, M. Blaut, O. Liesenfeld, R. R. Schumann, U. B. Göbel, and S. Bereswill. 2007. Exacerbation of murine ileitis by Toll-like receptor 4 mediated sensing of lipopolysaccharide from commensal *Escherichia coli*. *Gut* 56: 941–948.

69. Egan, C. E., K. J. Maurer, S. B. Cohen, M. Mack, K. W. Simpson, and E. Y. Denkers. 2011. Synergy between intraepithelial lymphocytes and lamina propria T cells drives intestinal inflammation during infection. *Mucosal Immunol* 4: 658–670.

70. Clevers, H. C., and C. L. Bevins. 2013. Paneth cells: maestros of the small intestinal crypts. *Annu. Rev. Physiol.* 75: 289–311.

71. Foureau, D. M., D. W. Mielcarz, L. C. Menard, J. Schulthess, C. Werts, V. Vasseur, B. Ryffel, L. H. Kasper, and D. Buzoni-Gatel. 2010. TLR9-dependent induction of intestinal alpha-defensins by *Toxoplasma gondii*. *The Journal of Immunology* 184: 7022–7029.

72. Raetz, M., S.-H. Hwang, C. L. Wilhelm, D. Kirkland, A. Benson, C. R. Sturge, J. Mirpuri, S. Vaishnav, B. Hou, A. L. DeFranco, C. J. Gilpin, L. V. Hooper, and F. Yarovinsky. 2013. Parasite-induced TH1 cells and intestinal dysbiosis cooperate in IFN- γ -dependent elimination of Paneth cells. *Nat. Immunol.* 14: 136–142.

73. Wehkamp, J., N. H. Salzman, E. Porter, S. Nuding, M. Weichenthal, R. E. Petras,

- B. Shen, E. Schaeffeler, M. Schwab, R. Linzmeier, R. W. Feathers, H. Chu, H. Lima, K. Fellermaier, T. Ganz, E. F. Stange, and C. L. Bevins. 2005. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc. Natl. Acad. Sci. U.S.A.* 102: 18129–18134.
74. Shi, C., and E. G. Pamer. 2011. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* 11: 762–774.
75. Serbina, N. V., T. P. Salazar-Mather, C. A. Biron, W. A. Kuziel, and E. G. Pamer. 2003. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 19: 59–70.
76. Serbina, N. V., and E. G. Pamer. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 7: 311–317.
77. Schulthess, J., B. Meresse, E. Ramiro-Puig, N. Montcuquet, S. Darche, B. Bègue, F. Ruemmele, C. Combadière, J. P. Di Santo, D. Buzoni-Gatel, and N. Cerf-Bensussan. 2012. Interleukin-15-dependent NKp46+ innate lymphoid cells control intestinal inflammation by recruiting inflammatory monocytes. *Immunity* 37: 108–121.
78. Khan, I. A., P. M. Murphy, L. Casciotti, J. D. Schwartzman, J. Collins, J. L. Gao, and G. R. Yeaman. 2001. Mice lacking the chemokine receptor CCR1 show increased susceptibility to *Toxoplasma gondii* infection. *J. Immunol.* 166: 1930–1937.
79. Goldszmid, R. S., P. Caspar, A. Rivollier, S. White, A. Dzutsev, S. Hieny, B. Kelsall, G. Trinchieri, and A. Sher. 2012. NK cell-derived interferon- γ orchestrates cellular dynamics and the differentiation of monocytes into dendritic cells at the site of infection. *Immunity* 36: 1047–1059.
80. Serbina, N. V., T. Jia, T. M. Hohl, and E. G. Pamer. 2008. Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* 26: 421–452.
81. Schariton-Kersten, T. M., G. Yap, and J. Magram. 1997. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen *Toxoplasma gondii*. *J Exp Med.* 185:1261-1273.
82. Schariton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, S. Hieny, R. T. Gazzinelli, and A. Sher. 1996. In the absence of endogenous IFN- γ , mice develop unimpaired IL-12 responses to *Toxoplasma gondii* while failing to control acute infection. *J. Immunol.* 157: 4045–4054.
83. Howard, J. C., J. P. Hunn, and T. Steinfeldt. 2011. The IRG protein-based resistance mechanism in mice and its relation to virulence in *Toxoplasma gondii*. *Curr. Opin. Microbiol.* 14: 414–421.

84. Khaminets, A., J. P. Hunn, S. Könen-Waisman, Y. O. Zhao, D. Preukschat, J. Coers, J. P. Boyle, Y.-C. Ong, J. C. Boothroyd, G. Reichmann, and J. C. Howard. 2010. Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell. Microbiol.* 12: 939–961.
85. Zhao, Y. O., A. Khaminets, J. P. Hunn, and J. C. Howard. 2009. Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFN γ -inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. *PLoS Pathog* 5: e1000288.
86. Martens, S., I. Parvanova, J. Zerrahn, G. Griffiths, G. Schell, G. Reichmann, and J. C. Howard. 2005. Disruption of *Toxoplasma gondii* parasitophorous vacuoles by the mouse p47-resistance GTPases. *PLoS Pathog* 1: e24.
87. Liesenfeld, O., I. Parvanova, J. Zerrahn, S.-J. Han, F. Heinrich, M. Muñoz, F. Kaiser, T. Aebischer, T. Buch, A. Waisman, G. Reichmann, O. Utermöhlen, E. von Stebut, F. D. von Loewenich, C. Bogdan, S. Specht, M. Saeftel, A. Hoerauf, M. M. Mota, S. Könen-Waisman, S. H. E. Kaufmann, and J. C. Howard. 2011. The IFN- γ -inducible GTPase, Irga6, protects mice against *Toxoplasma gondii* but not against *Plasmodium berghei* and some other intracellular pathogens. *PLoS ONE* 6: e20568.
88. Yamamoto, M., M. Okuyama, J. S. Ma, T. Kimura, N. Kamiyama, H. Saiga, J. Ohshima, M. Sasai, H. Kayama, T. Okamoto, D. C. S. Huang, D. Soldati-Favre, K. Horie, J. Takeda, and K. Takeda. 2012. A cluster of interferon- γ -inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. *Immunity* 37: 302–313.
89. Grainger, J. R., E. A. Wohlfert, I. J. Fuss, N. Bouladoux, M. H. Askenase, F. Legrand, L. Y. Koo, J. M. Brechley, I. D. C. Fraser, and Y. Belkaid. 2013. Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat. Med.* 19: 713–721.
90. Kalinski, P. 2012. Regulation of immune responses by prostaglandin E2. *The Journal of Immunology* 188: 21–28.
91. Kunisawa, J., I. Takahashi, and H. Kiyono. 2007. Intraepithelial lymphocytes: their shared and divergent immunological behaviors in the small and large intestine. *Immunol. Rev.* 215: 136–153.
92. Cheroutre, H., F. Lambolez, and D. Mucida. 2011. The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol* 11: 445–456.
93. Dalton, J. E., S. M. Cruickshank, C. E. Egan, R. Mears, D. J. Newton, E. M. Andrew, B. Lawrence, G. Howell, K. J. Else, M.-J. Gubbels, B. Striepen, J. E. Smith, S. J. White, and S. R. Carding. 2006. Intraepithelial $\gamma\delta$ lymphocytes maintain the integrity of intestinal epithelial tight junctions in response to infection.

Gastroenterology 131: 818–829.

94. Chardès, T., D. Buzoni-Gatel, A. Lepage, F. Bernard, and D. Bout. 1994. *Toxoplasma gondii* oral infection induces specific cytotoxic CD8 alpha/beta+ Thy-1+ gut intraepithelial lymphocytes, lytic for parasite-infected enterocytes. *J. Immunol.* 153: 4596–4603.

95. Buzoni-Gatel, D., H. Debbabi, F. J. Mennechet, V. Martin, A. C. Lepage, J. D. Schwartzman, and L. H. Kasper. 2001. Murine ileitis after intracellular parasite infection is controlled by TGF-beta-producing intraepithelial lymphocytes. *Gastroenterology* 120: 914–924.

96. Buzoni-Gatel, D., H. Debbabi, M. Moretto, I. H. Dimier-Poisson, A. C. Lepage, D. T. Bout, and L. H. Kasper. 1999. Intraepithelial lymphocytes traffic to the intestine and enhance resistance to *Toxoplasma gondii* oral infection. *J. Immunol.* 162: 5846–5852.

97. Luangsay, S., L. H. Kasper, N. Rachinel, L. A. Minns, F. J. D. Mennechet, A. Vandewalle, and D. Buzoni-Gatel. 2003. CCR5 mediates specific migration of *Toxoplasma gondii*-primed CD8 lymphocytes to inflammatory intestinal epithelial cells. *Gastroenterology* 125: 491–500.

98. Lepage, A. C., D. Buzoni-Gatel, D. T. Bout, and L. H. Kasper. 1998. Gut-derived intraepithelial lymphocytes induce long term immunity against *Toxoplasma gondii*. *J. Immunol.* 161: 4902–4908.

99. Mennechet, F. J. D., L. H. Kasper, N. Rachinel, W. Li, A. Vandewalle, and D. Buzoni-Gatel. 2002. Lamina propria CD4+ T lymphocytes synergize with murine intestinal epithelial cells to enhance proinflammatory response against an intracellular pathogen. *J. Immunol.* 168: 2988–2996.

100. Mennechet, F. J. D., L. H. Kasper, N. Rachinel, L. A. Minns, S. Luangsay, A. Vandewalle, and D. Buzoni-Gatel. 2004. Intestinal intraepithelial lymphocytes prevent pathogen-driven inflammation and regulate the Smad/T-bet pathway of lamina propria CD4+ T cells. *Eur. J. Immunol.* 34: 1059–1067.

101. Egan, C. E., S. B. Cohen, and E. Y. Denkers. 2012. Insights into inflammatory bowel disease using *Toxoplasma gondii* as an infectious trigger. *Immunol. Cell Biol.* 90: 668–675.

102. Egan, C. E., M. D. Craven, J. Leng, M. Mack, K. W. Simpson, and E. Y. Denkers. 2009. CCR2-dependent intraepithelial lymphocytes mediate inflammatory gut pathology during *Toxoplasma gondii* infection. *Mucosal Immunol* 2: 527–535.

103. Liesenfeld, O., J. Kosek, J. S. Remington, and Y. Suzuki. 1996. Association of

CD4⁺ T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with *Toxoplasma gondii*. *J. Exp. Med.* 184: 597–607.

104. Bollrath, J., and F. M. Powrie. 2013. Controlling the frontier: Regulatory T-cells and intestinal homeostasis. *Semin. Immunol.* 25: 352–357.

105. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kühn, W. Müller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4⁺ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J. Immunol.* 157: 798–805.

106. Suzuki, Y., A. Sher, G. Yap, D. Park, L. E. Neyer, O. Liesenfeld, M. Fort, H. Kang, and E. Gufwoli. 2000. IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with *Toxoplasma gondii*. *J. Immunol.* 164: 5375–5382.

107. Hall, A. O., D. P. Beiting, C. Tato, B. John, G. Oldenhove, C. G. Lombana, G. H. Pritchard, J. S. Silver, N. Bouladoux, J. S. Stumhofer, T. H. Harris, J. Grainger, E. D. T. Wojno, S. Wagage, D. S. Roos, P. Scott, L. A. Turka, S. Cherry, S. L. Reiner, D. Cua, Y. Belkaid, M. M. Elloso, and C. A. Hunter. 2012. The cytokines interleukin 27 and interferon- γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity* 37: 511–523.

108. Oldenhove, G., N. Bouladoux, E. A. Wohlfert, J. A. Hall, D. Chou, L. Dos Santos, S. O'Brien, R. Blank, E. Lamb, S. Natarajan, R. Kastenmayer, C. Hunter, M. E. Grigg, and Y. Belkaid. 2009. Decrease of Foxp3⁺ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* 31: 772–786.

109. Benson, A., S. Murray, P. Divakar, N. Burnaevskiy, R. Pifer, J. Forman, and F. Yarovinsky. 2012. Microbial infection-induced expansion of effector T cells overcomes the suppressive effects of regulatory T cells via an IL-2 deprivation mechanism. *The Journal of Immunology* 188: 800–810.

110. Roers, A., L. Siewe, E. Strittmatter, M. Deckert, D. Schlüter, W. Stenzel, A. D. Gruber, T. Krieg, K. Rajewsky, and W. Müller. 2004. T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *J. Exp. Med.* 200: 1289–1297.

111. Jankovic, D., M. C. Kullberg, C. G. Feng, R. S. Goldszmid, C. M. Collazo, M. Wilson, T. A. Wynn, M. Kamanaka, R. A. Flavell, and A. Sher. 2007. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J. Exp. Med.* 204: 273–283.

112. Ronet, C., S. Darche, M. Leite de Moraes, S. Miyake, T. Yamamura, J. A. Louis, L. H. Kasper, and D. Buzoni-Gatel. 2005. NKT cells are critical for the initiation of an inflammatory bowel response against *Toxoplasma gondii*. *J. Immunol.* 175: 899–908.
113. Perona-Wright, G., K. Mohrs, F. M. Szaba, L. W. Kummer, R. Madan, C. L. Karp, L. L. Johnson, S. T. Smiley, and M. Mohrs. 2009. Systemic but not local infections elicit immunosuppressive IL-10 production by natural killer cells. *Cell Host Microbe* 6: 503–512.

CHAPTER 3

CXCR3-Dependent CD4⁺ T cells are Required to Activate Inflammatory Monocytes for Defense Against Intestinal Infection*

*Adapted from: Cohen, SB, Maurer, KJ, Egan, CE, Oghumu, Satoskar, AR, Denkers, EY. “CXCR3-Dependent CD4⁺ T cells are Required to Activate Inflammatory Monocytes for Defense Against Intestinal Infection” *PLoS Pathogens*. 2013; 9(10): e1003706.

Abstract

Chemokines and their receptors play a critical role in orchestrating immunity to microbial pathogens, including the orally acquired Th1-inducing protozoan parasite *Toxoplasma gondii*. Chemokine receptor CXCR3 is associated with Th1 responses, and here we use bicistronic CXCR3-eGFP knock-in reporter mice to demonstrate upregulation of this chemokine receptor on CD4⁺ and CD8⁺ T lymphocytes during *Toxoplasma* infection. We show a critical role for CXCR3 in resistance to the parasite in the intestinal mucosa. Absence of the receptor in *Cxcr3*^{-/-} mice resulted in selective loss of ability to control *T. gondii* specifically in the lamina propria compartment. CD4⁺ T cells were impaired both in their recruitment to the intestinal lamina propria and in their ability to secrete IFN- γ upon stimulation. Local recruitment of CD11b⁺Ly6C/G⁺ inflammatory monocytes, recently reported to be major anti-*Toxoplasma* effectors in the intestine, was not impacted by loss of CXCR3. However, inflammatory monocyte activation status, as measured by dual production of TNF- α and IL-12, was severely impaired in *Cxcr3*^{-/-} mice. Strikingly, adoptive transfer of wild-type but not *Ifn γ* ^{-/-} CD4⁺ T lymphocytes into *Cxcr3*^{-/-} animals prior to infection corrected the defect in inflammatory macrophage activation, simultaneously reversing the susceptibility phenotype of the knockout animals. Our results establish a central role for CXCR3 in coordinating innate and adaptive immunity, ensuring generation of Th1 effectors and their trafficking to the frontline of infection to program microbial killing by inflammatory monocytes.

Introduction

The intestinal mucosa is a critical effector site for elimination of enteric pathogens. *Toxoplasma gondii*, a ubiquitous protozoan parasite, is a prime example of such a pathogen. Mammals are infected with *T. gondii* primarily by the ingestion of tissue cysts from undercooked meat or oocysts excreted in the feces of felines, which are the sole definitive hosts. Upon infection, the parasite induces a potent Th1 immune response that is characterized by high levels of IL-12 and IFN- γ (1, 2). Initial IL-12 production is largely the result of MyD88-dependent Toll-like receptor (TLR) signaling in dendritic cells, and the parasite profilin molecule has been identified as a ligand for TLR11 and TLR12 (3-7). IL-12 activates natural killer (NK) cells to initiate IFN- γ production and promotes T-cell differentiation towards a Th1 program. Ultimately IFN- γ is the critical cytokine involved in controlling *Toxoplasma*. While *in vitro* experiments suggest that macrophages activated by this cytokine acquire anti-*Toxoplasma* activity through upregulation of immunity-related GTPase (IRG) molecules that mediate destruction of the parasitophorous vacuole (8-10), the *in vivo* function of IFN- γ is less clear.

Inflammatory monocytes are an important component of defense against microbial pathogens, including *Toxoplasma* (11). These cells express high levels of Ly6C/G (Gr-1) and are recruited from the bone marrow via chemokine (C-C motif) receptor 2 (CCR2) (12). During *Listeria monocytogenes* infection, inflammatory monocytes are recruited from the bone marrow to the spleen and liver where they differentiate into TNF- α - and nitric oxide (NO)-producing DCs (Tip-DCs). There they are essential for

bacterial clearance and mouse survival (13, 14). Likewise, CCR2-dependent inflammatory monocytes are recruited to the lung during *Mycobacteria tuberculosis* infection where they protect mice from disease by recruiting and activating T cells and by producing NO (15, 16). Mucosal defense against *T. gondii* has also recently been shown to require CCR2-dependent inflammatory monocytes (11). Upon recruitment to the small intestine, these cells control the parasite either indirectly by production of IL-12 and TNF- α , or directly through production of NO and IRG proteins (4-6, 8, 9, 11, 17). While CCR2 enables recruitment of inflammatory monocytes to sites of infection, the factors that coordinate their activation and acquisition of effector function are not known.

CXCR3 is a Th1-associated chemokine receptor, and cells expressing this receptor respond to the IFN- γ -inducible chemokines CXCL9, 10, and 11 (18). The receptor is expressed predominantly by T cells and NK cells and is rapidly upregulated upon cell activation. There is evidence that CXCR3 expression enables T-cell entry into sites of infection, although the outcome of recruitment varies among pathogens. In the case of *Leishmania major*, recruitment is protective as CXCR3-expressing T cells are required for the resolution of cutaneous lesions (19). However, in the case of *Plasmodium berghei* ANKA, CXCR3 is pathogenic because it allows entry of proinflammatory cells into the CNS, resulting in cerebral malaria (20).

Here we determined the role of CXCR3 in the intestinal immune response to *Toxoplasma*. We found that loss of CXCR3 negatively affected host survival against

oral infection. This was associated with diminished recruitment of CD4⁺ T cells to the lamina propria (LP), decreased T cell IFN- γ secretion, impaired inflammatory monocyte effector function, and inability to control the parasite in the intestinal mucosa. Reconstitution with CXCR3-competent CD4⁺ T cells restored inflammatory monocyte function, resulting in improved survival against the parasite. Protective effects of adoptively transferred CD4⁺ T lymphocytes depended upon their ability to produce IFN- γ , but occurred independently of CD4 expression of CD40L. Our data show that CXCR3 enables Th1 recruitment to the intestinal LP, where these cells instruct activation of CCR2-dependent inflammatory monocytes, in turn controlling infection. These results establish CXCR3 as a major determinant orchestrating communication between effectors of innate and adaptive immunity, enabling effective host defense against infection.

Results

CXCR3 and its ligands CXCL9 and CXCL10 are upregulated during acute toxoplasmosis

Because CXCR3 and its chemokine ligands are strongly associated with Th1 responses, we asked whether this proinflammatory axis was induced during *Toxoplasma* infection in the intestinal mucosa. Accordingly, mice were orally inoculated with cysts, and relative levels of CXCR3, CXCL9 and CXCL10 mRNA expression were measured over the course of acute infection. We found strong upregulation of CXCR3 and its specific chemokine ligands as early as Day 4 post-infection in both the ileum and mesenteric lymph nodes (MLN) (Figure 3.1A). Overall, peak CXCR3 mRNA levels were attained by Day 6 post-inoculation.

In order to examine CXCR3 expression in more detail, we utilized *Cxcr3* eGFP reporter (CIBER) mice, a bicistronic reporter strain in which cells expressing CXCR3 also express eGFP (21). We found a large increase in CXCR3 populations of both CD4⁺ and CD8⁺ T lymphocytes in MLN, spleen (SPL) and LP compartments following infection (Figure 3.1B). In general, CXCR3 upregulation was most pronounced in the CD4⁺ population. For example, in the MLN there was a 6-fold increase in CD4⁺CXCR3⁺ cells but only a 2-fold increase in CD8⁺CXCR3⁺ lymphocytes. NK cells are also known to express CXCR3 and are an important source of early IFN- γ during *T. gondii* infection (22, 23). However, while CXCR3-GFP expression was relatively high on naïve NK cells, the GFP expression was in fact reduced during infection, suggesting lack of a role for CXCR3⁺ NK cells during

intestinal infection (Figure 3.2). We next examined expression of the activation marker CD27 on GFP⁺ and GFP⁻ T lymphocytes in infected mice. CD27 was significantly lower in CXCR3⁻GFP⁻ cells, suggesting an altered maturation state of the CXCR3⁻ T cells (Figure 3.3A and B). Likewise, there was a lower percentage of CD27⁺ cells amongst CXCR3-GFP⁻ CD8⁺ T lymphocytes, although the decreases in expression were not as striking as with the CD4⁺ lymphocytes (Figure 3.3C and D).

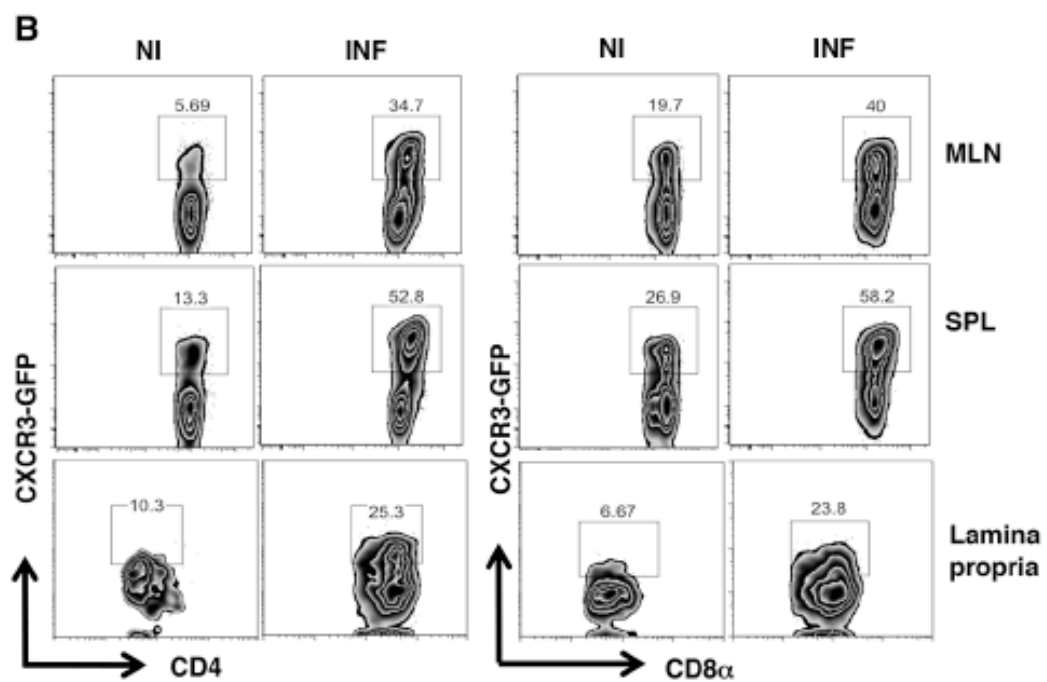
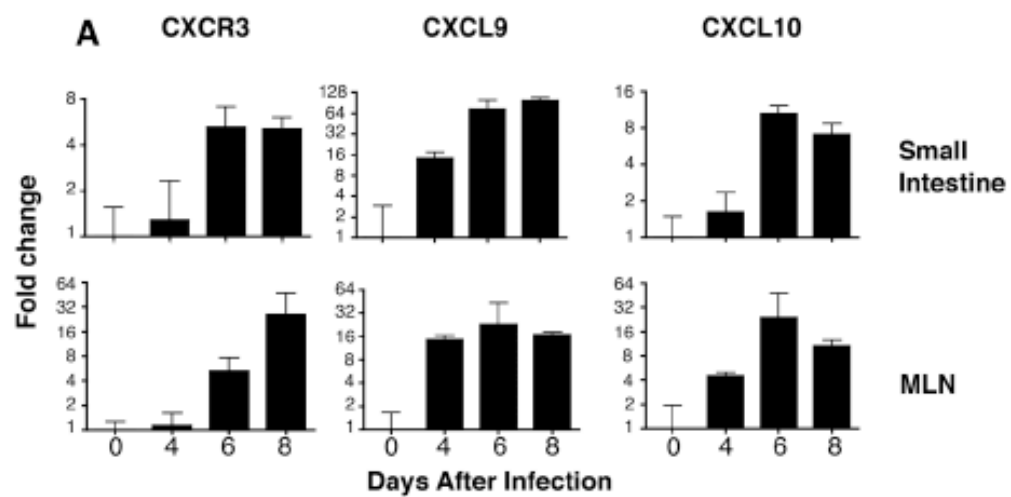


Figure 3.1. CXCR3 and its ligands are upregulated following *T. gondii* infection.

(A) CXCR3, CXCL9, and CXCL10 gene expression was assessed by semi-quantitative real time PCR in mesenteric lymph nodes (MLN) and small intestinal tissue from WT mice during oral *T. gondii* infection. The results are expressed as fold change relative to tissues from noninfected animals (n=4 mice per time point). (B) CXCR3 protein expression was quantified using flow cytometry by measuring GFP levels before (noninfected, NI) and 11 days after oral infection (INF) of CIBER mice. GFP levels were assessed among CD4⁺ and CD8⁺ T-cell subsets from MLN, spleen (SPL), and small intestinal lamina propria. NI, noninfected; INF, infected.

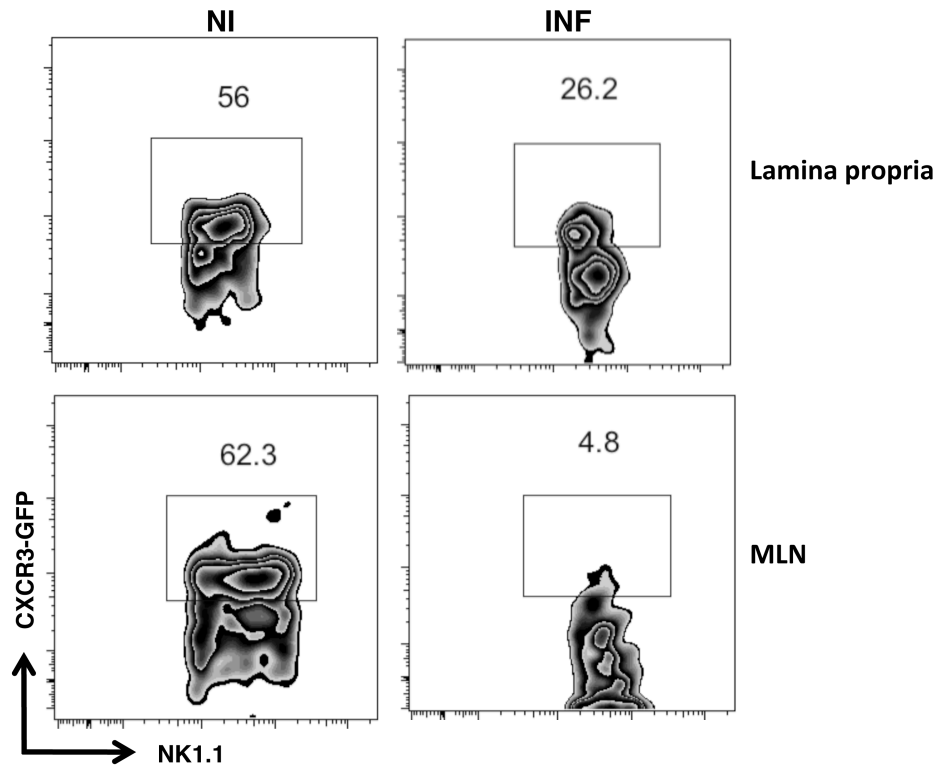


Figure 3.2 CXCR3-GFP expression on NK cells decreases during infection.

Lamina propria and mesenteric lymph node (MLN) leukocytes were isolated from noninfected (NI) and Day 7-infected (INF) CIBER reporter mice, and CXCR3-GFP expression was measured on NK1.1⁺ cells. Numbers in each panel indicate the percent of NK1.1⁺ cells falling within the CXCR3-eGFP positive quadrant.

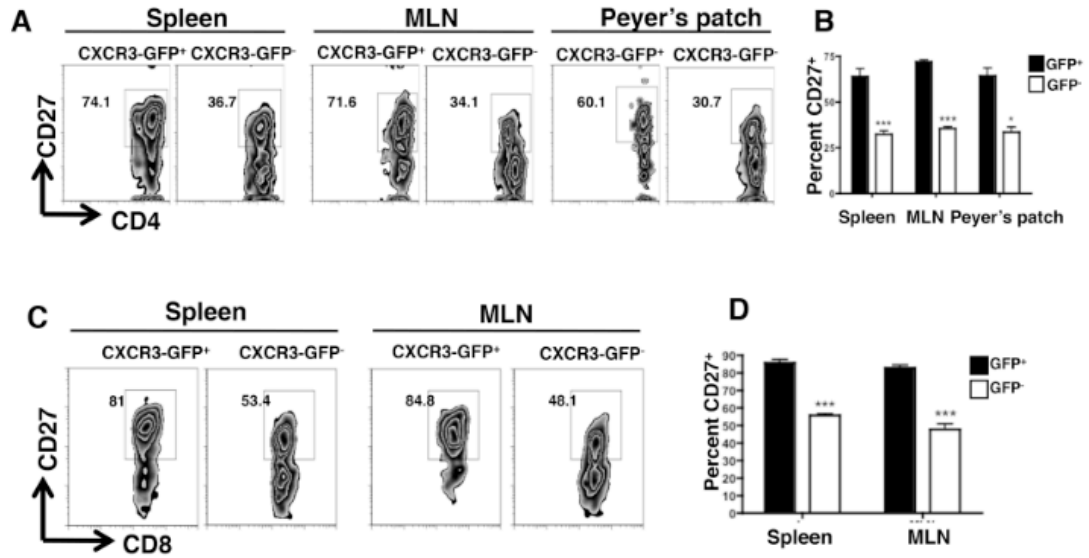


Figure 3.3. CXCR3-GFP⁺ and CXCR3-GFP⁻ fractions of CD4⁺ T cells differentially express activation markers. Splenocytes (n=4), MLN (n=5), and Peyer's patch (n=2) cells were isolated from Day 11-infected CIBER reporter mice and stained for surface markers CD27 as well as CD4 (A and B) and CD8 (C and D). Representative mice are shown in A and C. Bar graphs (B and D) represent averages of multiple mice, and significance is represented by * p<0.05, and *** p<0.001.

***Cxcr3*^{-/-} mice are increased in susceptibility and are prone to severe intestinal damage following *T. gondii* infection**

To further examine the role of CXCR3 during *T. gondii* infection, mice deficient in CXCR3 were orally inoculated with low virulence ME49 cysts, and the outcome of infection was monitored. While all wild-type (WT) mice survived acute infection with 30 cysts, *Cxcr3*^{-/-} animals displayed increased susceptibility with nearly 75% of mice dying by 2 weeks post-infection (Figure 3.4A). When the cyst dose was increased to 50, all CXCR3 knockout (KO) mice rapidly succumbed to infection, but some WT mice also died (Figure 3.4B). Interestingly, when WT and KO mice were infected by intraperitoneal injection, lack of CXCR3 did not affect survival, indicating that the effect of CXCR3 is specific to the mucosal response (Figure 3.5A). To further examine the overall response in orally infected mice, we examined the gross appearance of the small intestine of WT and *Cxcr3*^{-/-} mice after 30-cyst infection. The small intestines of the KO mice were strikingly damaged as demonstrated by massive hemorrhage compared to WT (Figure 3.4C). Consistent with intestinal shortening associated with increased damage (24-26), the length of the small intestine was reduced in the KO mice during infection (Figure 3.4D). Increased damage was further confirmed by H&E staining of small intestinal sections. WT mice displayed minor villus blunting accompanied by moderate to severe inflammatory cell recruitment in the submucosa (Figure 3.4E and G). In contrast, *Cxcr3*^{-/-} mice displayed severe villus blunting, fusion, epithelial necrosis, sloughing of villus tips, and vascular congestion and hemorrhage (Figure 3.4F and H). Blind scoring of H&E sections revealed a significant decrease in inflammation scores in the absence of CXCR3 (Figure 3.4I),

but when parameters of intestinal damage were quantitated, *Cxcr3*^{-/-} mice scored significantly higher than WT counterparts (Figure 3.4J). This damage was infection-dependent as intestines from non-infected WT and *Cxcr3*^{-/-} mice both had normal architecture with few inflammatory cells (Figure 3.5B). Increased epithelial damage in the absence of CXCR3 was further verified by loss of epithelial surface-associated Muc1 compared to infected WT animals, suggesting epithelial cell sloughing (Figure 3.5C). Despite the overall decreased inflammatory score, *Cxcr3*^{-/-} mice consistently displayed an influx of neutrophils into the LP compartment compared to WT mice, suggesting a role for these cells in causing damage, as argued by others (13, 14, 27) (Figure 3.5D and E).

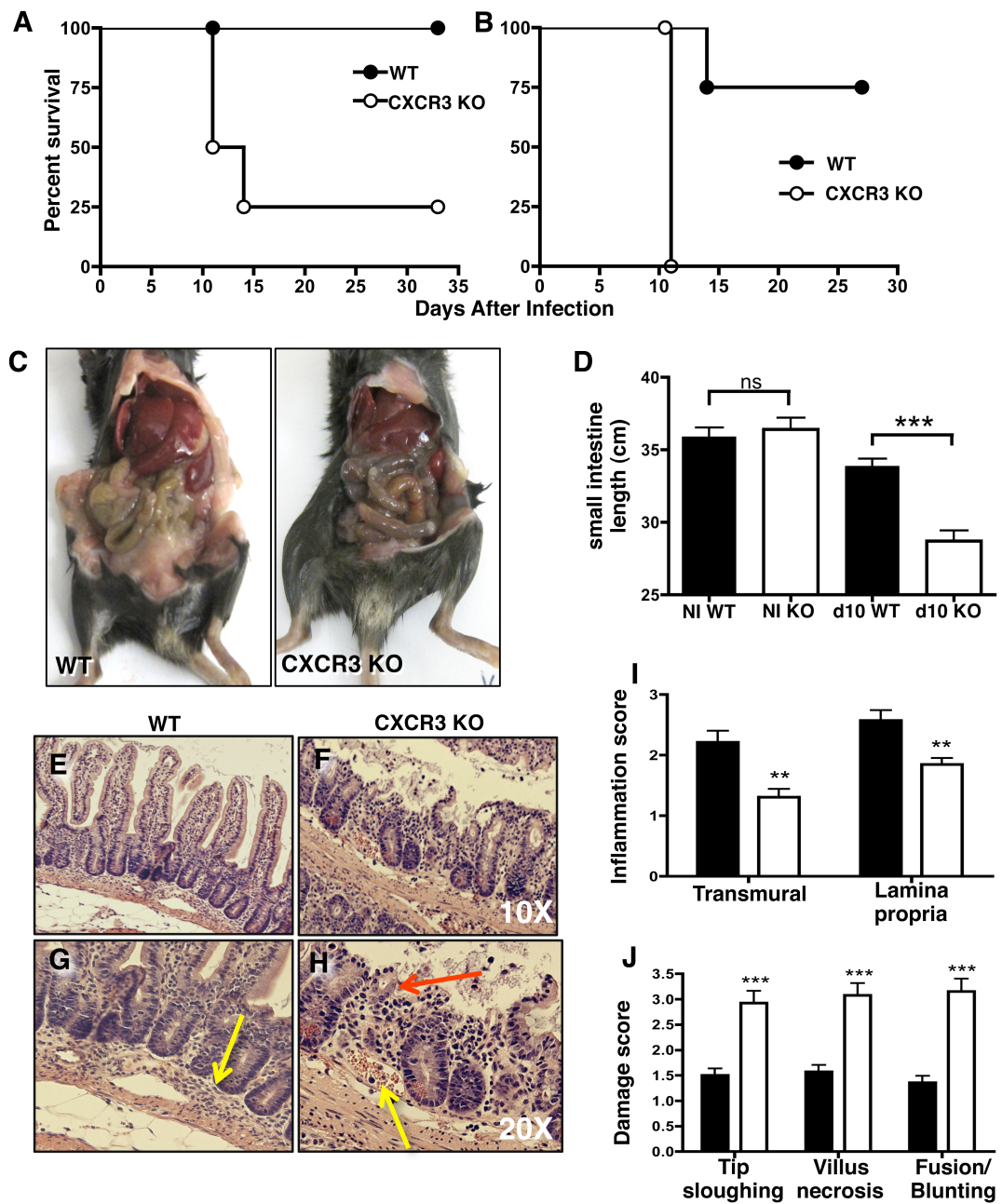


Figure 3.4. *Cxcr3*^{-/-} mice are susceptible to severe intestinal pathology following oral *T. gondii* infection. WT and CXCR3-deficient mice were orally inoculated with 30 ME49 cysts (p<0.01) (A) or 50 cysts (p<0.0001) (B) of *T. gondii* and monitored for survival. In another set of experiments (C-J), mice were orally inoculated with 30 ME49 cysts, and tissues were collected at Day 10 post-infection. (C) Gross intestinal lesions in representative WT and CXCR3 KO mice. (D) Average lengths of noninfected (NI) and infected (INF) WT and *Cxcr3*^{-/-} small intestines (NI WT, n=5; NI KO, n=3; INF WT, n=8, INF KO: n=7). E-H, H&E stained sections of small intestines from infected WT (E and G) and KO (F and H) mice. In panel G, the arrow points to an area of inflammatory cell influx. In panel H, the yellow arrow indicates an area of vascular congestion, and the red arrow indicates a necrotic villus. Blind scoring was performed on H&E stained intestine sections for inflammation (I) and damage (J) criteria (WT: n=14; KO: n=13; * p<0.05, ** p<0.01, *** p<0.001). Pooled data are represented as mean +/- SEM.

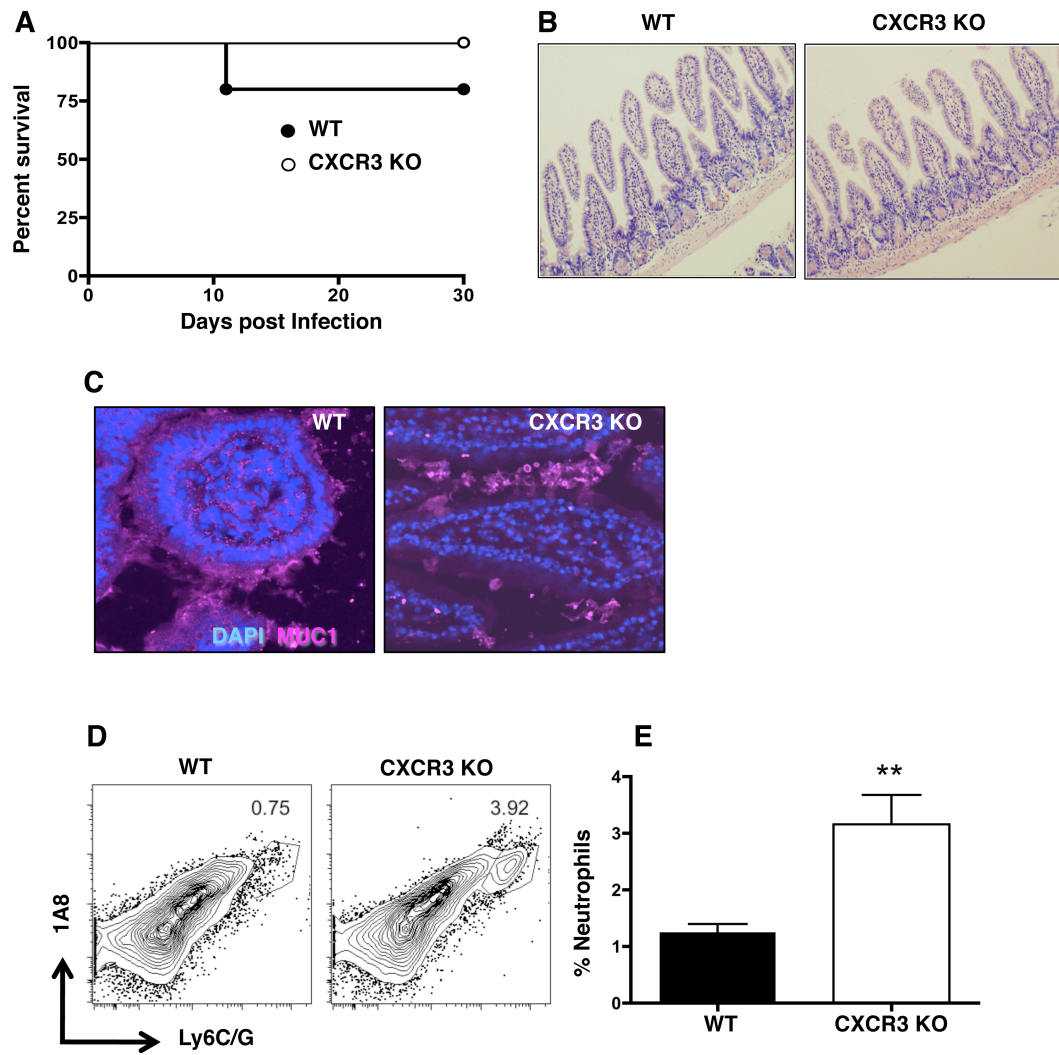


Figure 3.5. *Cxcr3*^{-/-} mice are more susceptible to oral infection with *Toxoplasma*.

(A) WT and KO mice (n=5 per group) were infected by i.p. injection of 30 ME49 cysts, and survival was monitored (p=0.371). (B) Small intestines of naïve WT and KO mice (n=3 per strain) were harvested, fixed in formaldehyde, embedded in paraffin, and sections were stained with H&E. (C) Frozen sections of Day 10 WT and *Cxcr3*^{-/-} intestines were stained for Muc1 followed by anti-rabbit Alexa-488 (red). Sections were counter-stained with DAPI (blue). (D) Lamina propria leukocytes from Day 9-orally infected WT and *Cxcr3*^{-/-} mice were stained for neutrophil markers Ly6C/G (Gr-1) and Ly6G (1A8). (E) Neutrophil levels were assessed in individual mice (n=5 per group). The graph shows mean +/- SEM (** p<0.01).

***Cxcr3*^{-/-} mice are unable to control parasite replication in the small intestine**

Genetic knockout of cytokines such as IFN- γ results in susceptibility to *T. gondii* through the inability to control parasite replication, whereas the deletion of anti-inflammatory mediators such as IL-10 results in susceptibility due to cytokine pathology (28, 29). Based on decreased inflammation scores, we hypothesized that the *Cxcr3*^{-/-} mice were more likely to be succumbing from uncontrolled parasite replication rather than immune-mediated damage. To examine this, intestinal tissues were stained for parasite antigen by immunohistochemistry. Sections from WT mice displayed minimal parasite infiltration within the LP (Figure 3.6A). Conversely, *Cxcr3*^{-/-} mice contained numerous large foci of parasite throughout the length of the small intestine that often coincided with areas of severe damage (Figure 3.6B). Surprisingly, this difference was restricted to the LP and submucosa of the small intestine because Peyer's patches (PP) in WT and *Cxcr3*^{-/-} mice contained similar levels of parasite antigen (Figure 3.6C and D). Differences in parasite burden between WT and *Cxcr3*^{-/-} mice in the MLN, spleen, and lung were also indiscernible by IHC analysis (data not shown). These results were further confirmed by quantitative PCR. Thus, while lung, liver, spleen, MLN and PP contained similar levels of parasite genomes regardless of CXCR3 expression, there was an approximately 50-fold increase in parasite levels in the absence of CXCR3 in intestinal tissues (Figure 3.6E). These data suggest that increased susceptibility to *Toxoplasma* in *Cxcr3*^{-/-} mice was due to a localized inability to control parasite replication within the LP of the small intestine.

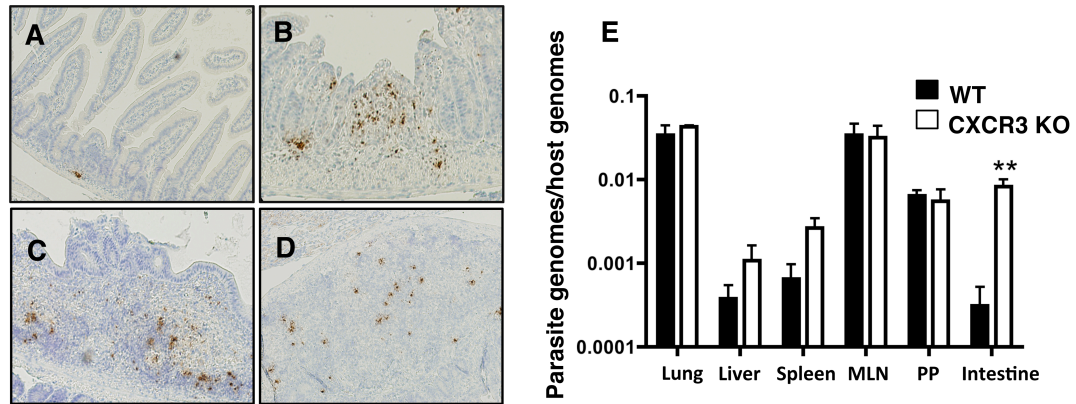


Figure 3.6. Intestinal parasite burden is elevated in *Cxcr3*^{-/-} mice. Paraffin sections of Day 10-infected WT and *Cxcr3*^{-/-} small intestines were stained by immunohistochemistry for *T. gondii* antigen. Shown are representative images of WT LP (A), *Cxcr3*^{-/-} LP (B), WT Peyer's patch (C), and *Cxcr3*^{-/-} Peyer's patch (D) with positive parasite staining in brown. (E) Lung (WT: n=3; KO: n=4), liver (WT: n=5; KO: n=4), spleen (WT: n=5; KO: n=4), MLN (WT: n=3; KO: n=5), Peyer's patches (PP, WT: n=2, KO: n=2) and small intestines (WT: n=5; KO: n=4) were harvested during acute infection, and DNA was isolated from tissues and subjected to quantitative PCR amplification for the parasite B1 gene and the host argininosuccinate lyase gene. Parasite burden was quantitated as parasite to host genome equivalents and was calculated by comparison to a standard curve obtained from known amounts of *Toxoplasma*. Pooled ratios are represented as mean +/- SEM where **p<0.01.

CD4⁺ T cells are recruited to the small intestine via CXCR3

The dominant effector cells required for elimination of *T. gondii* following oral infection are inflammatory monocytes. These cells express Ly6C/G (Gr-1), produce TNF- α , IL-12, and are likely to kill parasites via activation of IFN- γ -inducible p47 GTPases that assemble at the parasitophorous vacuole membrane and mediate its destruction (8, 11). Consistent with others (11), we observed these cells in the LP of infected mice (Figure 3.7A). Inflammatory monocytes are dependent upon CCR2 for exit from the bone marrow, but we wondered whether CXCR3 might be involved in recruiting these cells to the LP in response to *T. gondii*. Therefore, we examined CXCR3-GFP expression by intestinal inflammatory monocytes during infection. Inflammatory monocytes in the small intestinal LP of infected reporter mice did not express any GFP as compared to inflammatory monocytes isolated from infected *Cxcr3*^{-/-} mice (Figure 3.7B). In stark contrast, approximately 50% of LP CD4⁺ T cells expressed high levels of GFP (Figure 3.7C). Furthermore, *Cxcr3*^{-/-} mice displayed unaltered total numbers of LP inflammatory monocytes compared to wild-type controls (defined as CD11b⁺Ly6C⁺Ly6G⁻) (Figure 3.7D). We next assessed the kinetics by which CD4⁺ T cells and inflammatory monocytes were recruited to the lamina propria during infection. Between days 4 and 7 of infection, there was a significant increase in the total numbers of CD4⁺CXCR3⁻GFP⁺ T cells and inflammatory monocytes (Figure 3.7E). However, the total number of CD4⁺CXCR3⁻GFP⁻ cells remained unchanged, further indicating that infection promotes the recruitment of CD4⁺CXCR3⁺ T cells (Figure 3.7E). Few NK cells were observed in the lamina propria, but there was a small increase in their number during infection.

This was attributable to an increase in CXCR3⁺ NK cells (data not shown). Consistent with these results, there was an influx of CD4⁺ T cells in WT small intestines that was diminished in *Cxcr3*^{-/-} mice (Figure 3.7F-H). These findings demonstrate that CD4⁺ T cells fail to effectively traffic to the intestinal compartment in the absence of CXCR3, but the presence of LP inflammatory monocytes does not require this chemokine receptor.

Lamina propria CD4⁺ T cells display impaired IFN- γ production

We next asked if expression of CXCR3 affected the ability of T cells to secrete the Th1 cytokine IFN- γ . Initial experiments on bulk populations of splenocytes and mesenteric lymph node (MLN) cells from Day-11 infected WT and KO revealed no differences in the amount of IFN- γ , TNF- α or IL-10 produced during *in vitro* culture (Figure 3.8). To specifically examine functional outcomes in intestinal cells, WT and *Cxcr3*^{-/-} lamina propria leukocytes were harvested 4 and 6 days post-oral infection, stimulated *ex vivo*, and IFN- γ production was examined by flow cytometry. CD4⁺ T cells from both WT and *Cxcr3*^{-/-} displayed enhanced IFN- γ production over time. However, in the absence of CXCR3, CD4⁺ T cells produced significantly less IFN- γ compared to WT cells at both examined time points (Figure 3.9A-E). This was confirmed by measuring IFN- γ from the supernatants of Day-6 WT and *Cxcr3*^{-/-} intestinal biopsy cultures, where IFN- γ was lower in the absence of CXCR3 (Figure 3.9F). This effect was specific to the CD4⁺ T cell subset, as IFN- γ production by lamina propria CD8⁺ T cells and NK cells was unchanged between WT and knockout

animals (Figure 3.10A-F). Further confirming that this loss of IFN- γ production was specific to CD4⁺ T lymphocytes in the small intestine, and consistent with the bulk splenocyte culture experiments, splenocytes isolated from infected WT and *Cxcr3*^{-/-} mice secreted equivalent levels of IFN- γ upon *ex vivo* stimulation with PMA and ionomycin (Figure 3.10G-I). Together, these results indicate an intestine-specific defect in presence of CD4⁺ Th1 cells in the absence of CXCR3 as measured by the capacity to produce IFN- γ .

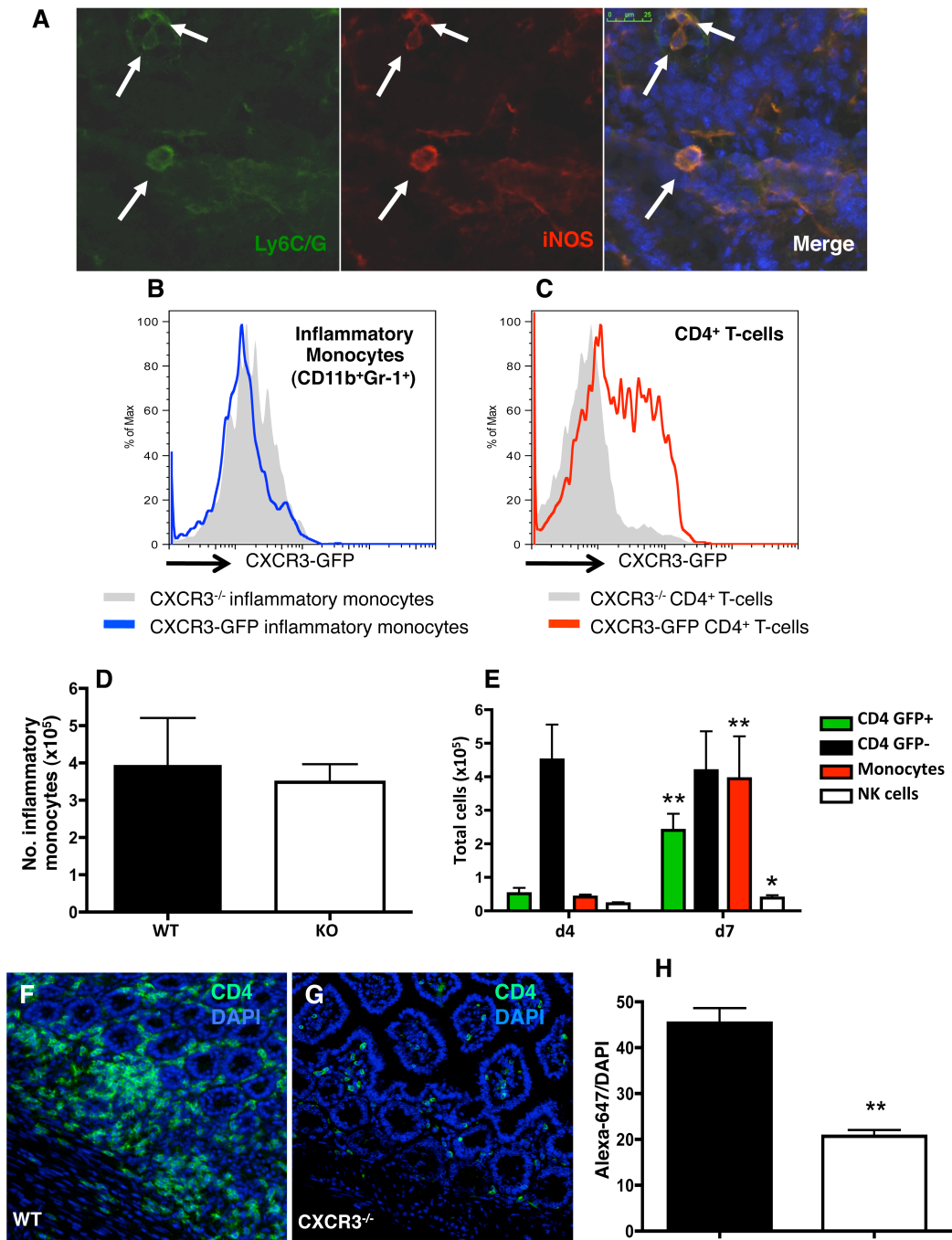


Figure 3.7. CD4⁺ T-cell recruitment, but not the presence of inflammatory monocytes, is impaired in the small intestine in the absence of CXCR3. (A) Frozen sections of intestines from infected WT mice were co-stained for Ly6C/G (Gr-1) (green) and iNOS (red) to confirm the presence of inflammatory monocytes in the mucosa of Day 6-infected animals. (B and C) Small intestinal LP cells were isolated from CXCR3-GFP reporters and *Cxcr3*^{-/-} mice 6 days following oral infection. In the CXCR3 reporter mice, inflammatory monocytes (B, blue line) and CD4⁺ T cells (C, red line) were assessed for GFP expression by flow cytometry as compared to *Cxcr3*^{-/-} cells (gray shaded in both histograms). (D) Total numbers of lamina propria inflammatory monocytes 6 days after infection. Neutrophils were excluded by gating on Ly6G-negative cells. (E) Total numbers of CD4⁺CXCR3-GFP⁺ T cells, CD4⁺CXCR3-GFP⁻ T cells, inflammatory monocytes, and NK cells in the lamina propria of WT and *Cxcr3*^{-/-} mice 4 and 7 days post-infection. Statistical comparisons were made between time points of respective cell types, where * p <0.05 and ** p <0.01. In panels F-G, WT (F) and *Cxcr3*^{-/-} (G) intestinal frozen sections were stained with anti-CD4 antibody followed by anti-rat Alexa-647. Sections were visualized by immunofluorescence microscopy. (H) To quantify CD4⁺ T-cell infiltration, the ratio of Alexa-647 over DAPI fluorescence was calculated (WT: n=3; KO: n=3; 6-12 fields/mouse; p<0.01). Pooled ratios are represented as mean +/- SEM.

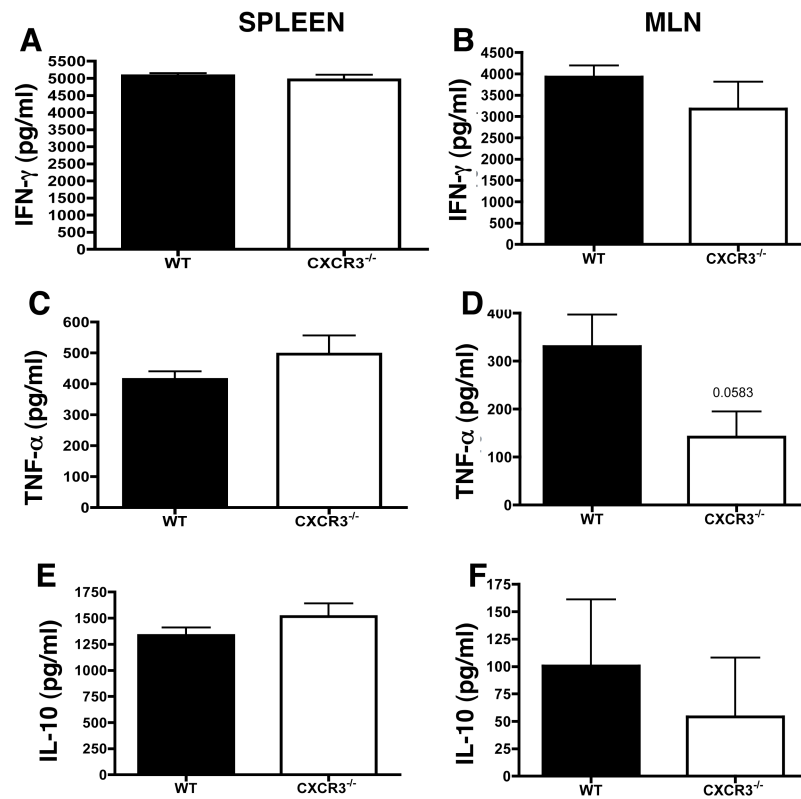


Figure 3.8. Cytokine responses in WT and KO mice. Splenocytes (A, C and E) and MLN (B, D and F) were harvested from Day 11-infected WT and *Cxcr3*^{-/-} mice and cultured in the presence of soluble tachyzoite antigen (STAg) for 72 hr. Supernatants were collected, and IFN-γ (A and B), TNF-α (C and D), and IL-10 (E and F) were measured by ELISA.

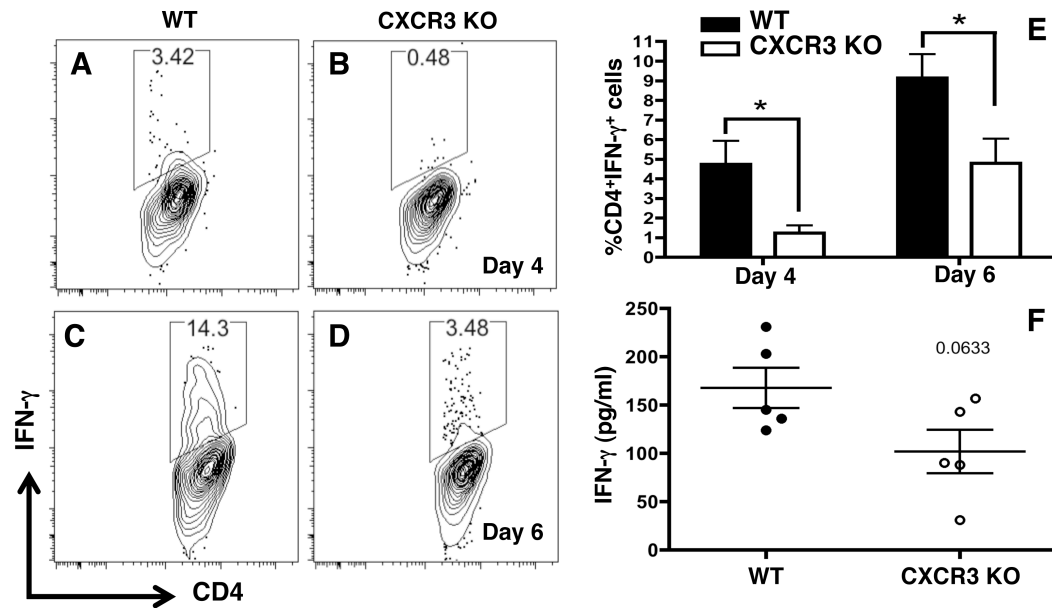


Figure 3.9. Lamina propria CD4⁺ T cells display impaired IFN- γ production.

Lamina propria leukocytes were harvested from WT and *Cxcr3*^{-/-} mice at Day 4 (A-B) and Day 6 (C-D) post-infection, cultured in the presence of PMA, ionomycin, and Brefeldin-A for 6 hrs, and assessed for IFN- γ production by flow cytometry. The means and standard errors of individual mice are shown in E (Day 4, n=10 per strain; Day 6 n=6 per strain). Intestinal biopsy cultures were performed at Day-6 post-infection, and IFN- γ was measured in the supernatants after 24 hr of culture (F). Each dot represents an individual mouse, and *p<0.05.

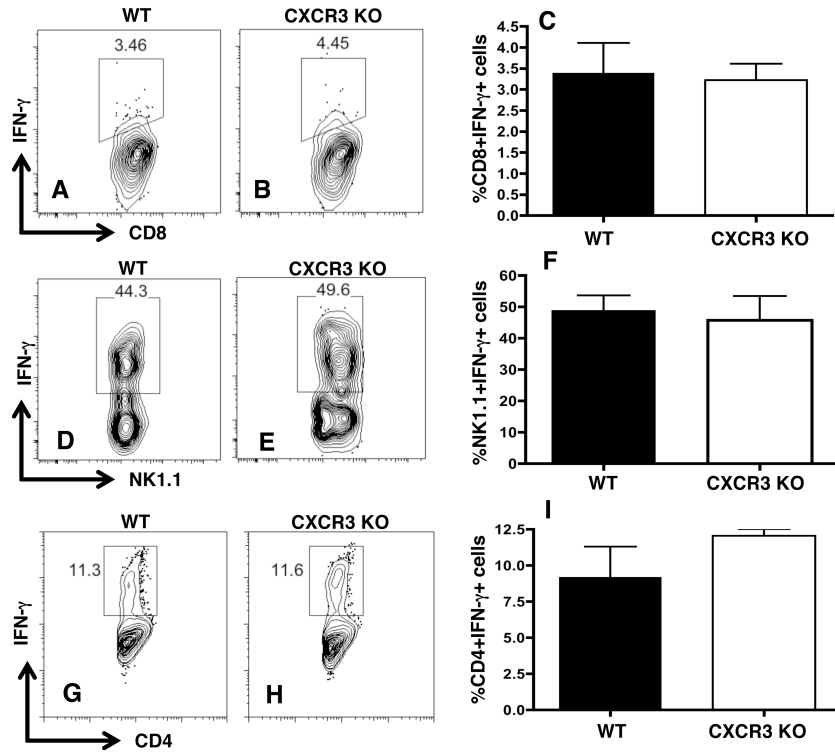


Figure 3.10. T cell and NK cell production of IFN- γ in the presence and absence of CXCR3. Lamina propria leukocytes were isolated from WT (A) and *Cxcr3*^{-/-} (B) mice 6 days post-infection, cultured for 6 hr in the presence of PMA, ionomycin, and Brefeldin-A, and stained for CD8 and IFN- γ . (C) Means and standard errors of individual mice. Lamina propria leukocytes were isolated from WT (D) and *Cxcr3*^{-/-} (E) mice, cultured in the presence of PMA, ionomycin, and Brefeldin-A and stained for NK1.1 and IFN- γ . (F) Mean and standard error of individual mice (WT: n=9; KO: n=6). Splenocytes were harvested from WT (G) and *Cxcr3*^{-/-} (H) Day 7-infected mice, cultured in the presence of PMA, ionomycin, and Brefeldin-A, and stained for CD4 and IFN- γ . (I) Mean and standard error of individual mice (WT: n=2; KO: n=2).

Inflammatory monocyte responses in the intestinal mucosa are defective in the absence of CXCR3

Although IFN- γ , IL-10, and TNF- α responses remained intact in the MLN and spleen late during infection of CXCR3-deficient mice, a significant decrease in IL-12 production was observed in the MLN (Figure 3.11A) and spleen (Figure 11B) of *Cxcr3*^{-/-} mice. Defective IL-12 responses in the CXCR3 KO strain were infection dependent, because parasite antigen stimulated equivalent amounts of IL-12 in noninfected WT and KO splenocytes (Figure 3.11C). This response, known to derive from resident splenic CD8 α ⁺ DC (30), may account for equivalent Th1 priming in secondary lymphoid organs, despite lower IL-12 levels during late acute infection.

Since IL-12 is also a characteristic cytokine of inflammatory monocytes, we investigated the impact of CXCR3 deletion on intestinal inflammatory monocyte function. Indeed, *in vitro* culture of intestinal biopsy samples revealed decreased production of IL-12 (Figure 3.11D). To further identify the source of the defective IL-12, we examined the production of IL-12 from inflammatory monocytes. While the total numbers of LP inflammatory monocytes were equivalent in WT and CXCR3 KO mice (Figure 3.7B), the population of CD11b⁺Gr-1⁺ cells co-expressing IL-12 and TNF- α was dependent upon CXCR3 (Figure 3.11E and F). Further confirming impaired inflammatory monocyte function, iNOS expression was significantly decreased in inflammatory monocytes (Figure 3.11G and H). These findings strongly suggest that inflammatory monocytes are functionally impaired in the absence of

CXCR3. Interestingly, neutrophils in the LP of KO mice produced significantly higher levels of TNF- α compared to WT neutrophils (Figure 3.12A and B).

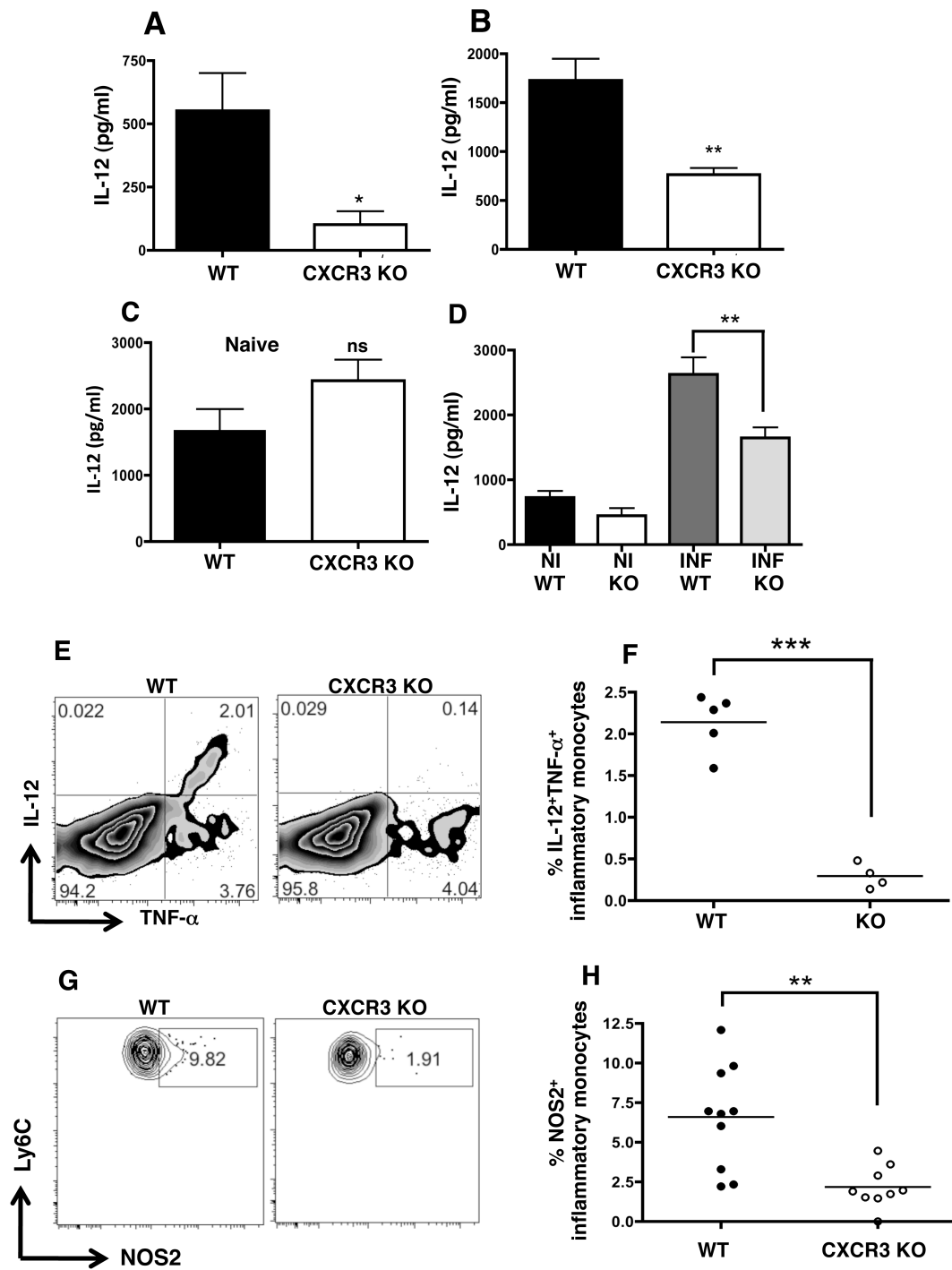


Figure 3.11. Inflammatory monocyte function is impaired in the absence of CXCR3. MLN cells (A) and splenocytes (B) from Day 11-infected WT and *Cxcr3*^{-/-} mice were cultured for 72 hours in complete DMEM in the presence of soluble tachyzoite antigen (STAg) (n=5 mice per strain). Supernatants were then collected and assayed for IL-12p40 by ELISA. (C) Naïve splenocytes were cultured with soluble tachyzoite lysate for 48h, and culture supernatants were assayed for IL-12p40 secretion. (D) Intestinal biopsy samples from noninfected (NI) and Day 9-infected (INF) WT and *Cxcr3*^{-/-} mice were cultured overnight, then supernatants were collected and assayed for IL-12p40. (WT: n=18; KO: n=16). Data are represented as mean +/- SEM. (E) Lamina propria leukocytes from Day 9-infected WT and *Cxcr3*^{-/-} mice were cultured in the presence of Brefeldin-A for 6 hr. Cells were surface stained for CD11b, Ly6G (1A8), and Ly6C/G (Gr-1) then intracellularly stained for IL-12 and TNF- α . The cell populations shown are gated on CD11b⁺Gr-1⁺1A8⁻ cells, and the quadrants represent proportions of cells positive for each cytokine. (F) IL-12⁺TNF- α ⁺ inflammatory monocyte levels in the lamina propria of individual mice. Each dot represents results from a single mouse. (G) Leukocytes were isolated from the MLN of Day 4-infected WT and *Cxcr3*^{-/-} animals and stained for iNOS by flow cytometry. (H) The means of multiple mice are plotted (WT: n=10; KO: n=9). In this figure, * p<0.05, ** p<0.01, *** p<0.001.

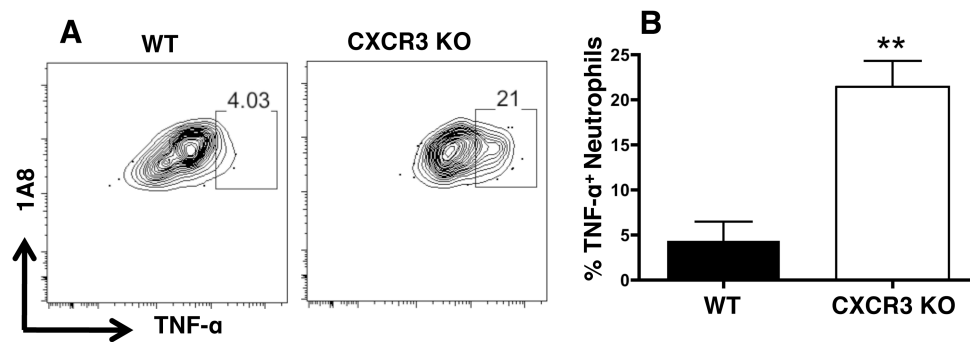


Figure 3.12. Lamina propria neutrophils secrete elevated TNF- α in the absence of CXCR3. Lamina propria leukocytes were harvested from WT and *Cxcr3*^{-/-} mice and cultured in the presence of Brefeldin-A for 6 hr. Cells were then stained for neutrophil markers CD11b, Ly6C/G (Gr-1), and Ly6G (1A8), fixed, permeabilized, and intracellularly stained for TNF- α . Cytokine production was assessed by flow cytometry (A). Shown are the mean \pm SEM of individual mice (B) (n=5 per group, ** p<0.01).

Adoptive transfer of WT CD4⁺ T lymphocytes rescues inflammatory monocyte function and restores resistance in *Cxcr3*^{-/-} mice

Given the data so far, we hypothesized that CD4⁺ T cells were unable to effectively home to the small intestine and prime inflammatory monocyte function in the absence of CXCR3, resulting in susceptibility to *Toxoplasma*. We therefore tested whether reconstitution with CXCR3-competent CD4⁺ T cells would allow *Cxcr3*^{-/-} mice to overcome susceptibility and restore inflammatory monocyte function. Accordingly, CD4⁺ T cells from naïve WT spleens were enriched to 90-95% purity by magnetic bead separation (Figure 3.13A) and injected i.v. into *Cxcr3*^{-/-} recipients. Mice were orally challenged with *T. gondii* 24 hours post-adoptive transfer, and survival was monitored. Knockout mice that did not receive WT cells began to die 10 days post-challenge, while all KO mice that received CD4⁺ T cells and all WT controls survived the acute phase of infection (Figure 3.13B). To confirm that the CD4-dependent survival was not an artifact of the transfer, *Cxcr3*^{-/-} CD4⁺ T cells were adoptively transferred into KO recipients. The knockout cells were unable to protect against susceptibility, demonstrating that protection is dependent on CXCR3 expression by CD4⁺ T cells (Figure 3.14A).

To assess the functional impact of WT CD4 adoptive transfer, parasite burden and cytokine production in the LP were assessed by flow cytometry in WT, *Cxcr3*^{-/-}, and *Cxcr3*^{-/-} + CD4 mice. While *Cxcr3*^{-/-} mice had a clear increase in *Toxoplasma* infected cells relative to WT, upon adoptive transfer of WT CD4⁺ T cells, parasite levels were reduced to WT (Figure 3.13C). Likewise, expression of IL-12/TNF- α by

inflammatory monocytes was significantly reduced in KO mice, but these cytokines returned to WT levels upon transfer of WT CD4⁺ T cells (Figure 3.13D and Figure 3.14B). In addition to cytokine responses, intestinal damage was also alleviated by adoptive transfer of WT CD4⁺ T cells, as the intestinal lengths of the transferred mice were restored to WT (Figure 3.13E). Possibly as a result of improved monocyte function and parasite clearance, neutrophil levels and neutrophil TNF- α secretion were also restored to WT levels following the transfer of WT CD4⁺ T cells (Figure 3.14C and D).

CD4⁺ T-cell rescue is dependent on IFN- γ but independent of CD40L

To identify the mechanism behind the rescue of *Cxcr3*^{-/-} susceptibility by WT CD4⁺ T cells, we performed adoptive transfer experiments utilizing T cells derived from knockout animals. Inasmuch as CXCR3 is a Th1 chemokine receptor, we began by asking whether reversal of susceptibility was dependent on CD4-derived IFN- γ . Therefore, CD4⁺ T cells were isolated from IFN- γ ^{-/-} mice and adoptively transferred into CXCR3-deficient recipients. Unlike IFN- γ -competent CD4⁺ T cells (Figure 3.13B and Figure 3.15B), transfer of IFN- γ KO CD4⁺ T lymphocytes failed to provide significant protection (Figure 3.15A). It has been shown that CD40L contributes to inflammatory responses in the intestinal mucosa during oral *Toxoplasma* infection (31). Therefore, we performed the adoptive transfer using CD40L^{-/-} CD4⁺ T cells and assessed survival. As expected, CXCR3-deficient animals were highly susceptible to infection. However, *Cxcr3*^{-/-} mice receiving CD40L^{-/-} CD4⁺ T cells survived the infection, indicating that CD40L does not mediate CXCR3⁺CD4⁺-dependent

protection (Figure 3.15B).

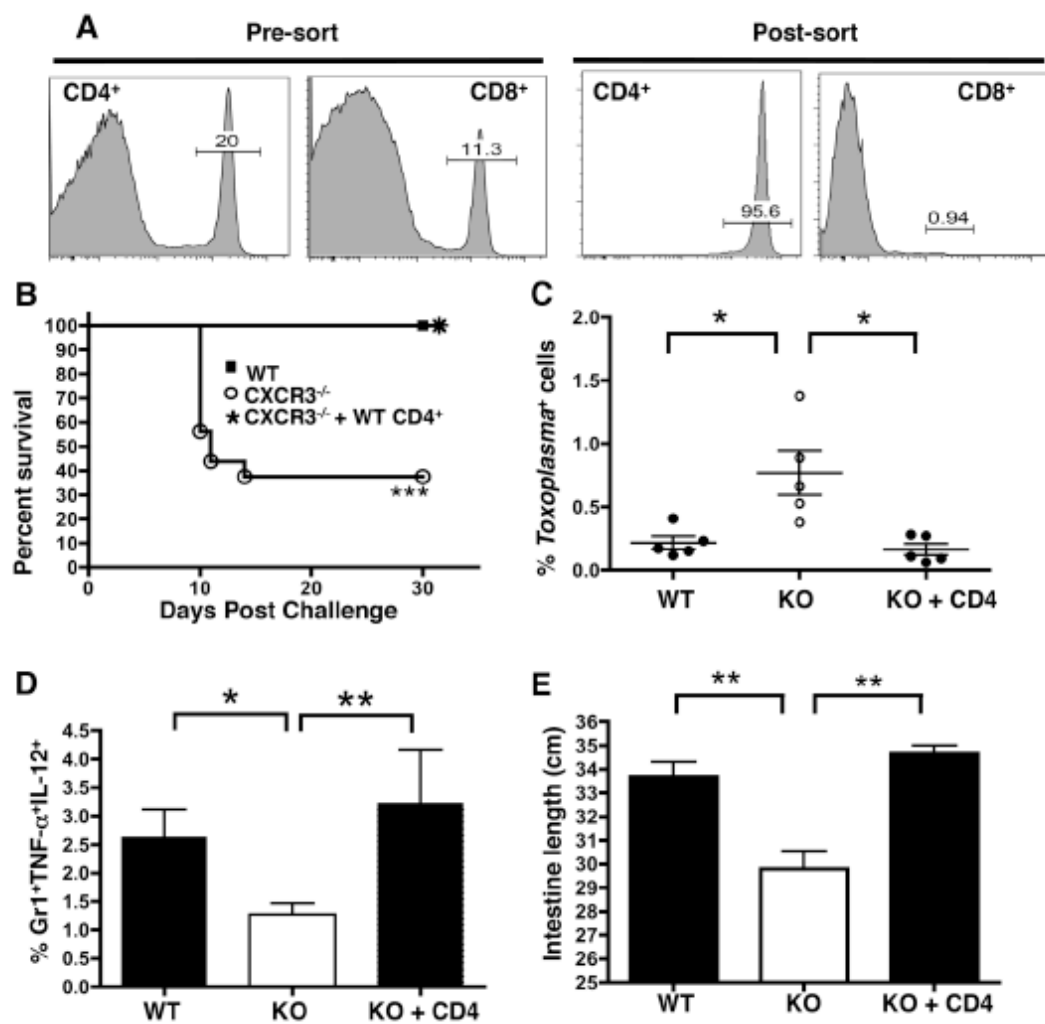


Figure 3.13. Adoptive transfer of WT CD4⁺ T cells into *Cxcr3*^{-/-} mice confers resistance to infection. (A) Splenocytes from naïve WT mice were sorted for CD4⁺ T cells by magnetic bead separation. Pre-sort and post-sort fractions were stained for CD4 and CD8 to confirm efficacy of the sort. (B) Purified CD4⁺ T cells (5x10⁶) from noninfected mice were adoptively transferred by intravenous injection into *Cxcr3*^{-/-} recipients (n=5 mice per group). Mice were orally challenged 24 hr post-transfer with *T. gondii* (30 cysts), and survival was monitored. ***, p<0.001 comparing *Cxcr3*^{-/-} with *Cxcr3*^{-/-} + CD4⁺ T cells. (C-E) Lamina propria leukocytes were isolated from WT, *Cxcr3*^{-/-} controls, and *Cxcr3*^{-/-} CD4⁺ T-cell recipients at Day 9 post-infection (n=5 mice per group). (C) Total cells were stained for *Toxoplasma* surface antigen (SAG)-1 to determine parasite infection. (D) Cells were also cultured in the presence of Brefeldin-A for 6 hours, after which they were surface stained for CD11b, Gr-1, and Ly6G and stained intracellularly for IL-12 and TNF- α . (E) Small intestinal length was compared between WT, *Cxcr3*^{-/-}, and *Cxcr3*^{-/-} adoptive transfer recipient mice (WT: n=11; KO: n=10; Transfer: n=3). Data are represented as mean +/- SEM, where * p<0.05 and ** p<0.01

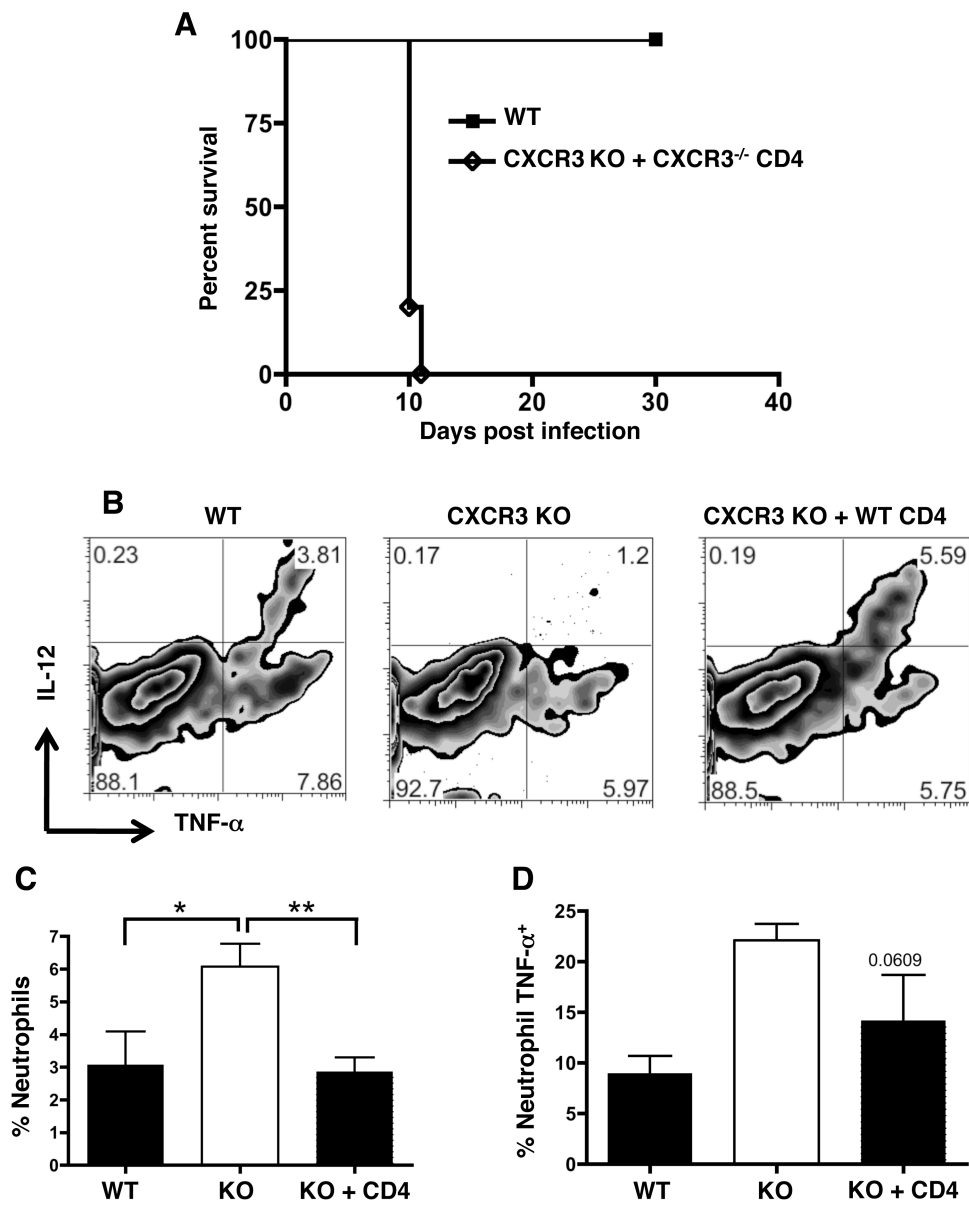


Figure 3.14. Adoptive transfer of CXCR3⁺CD4⁺ T cells into *Cxcr3*^{-/-} recipients protects against oral *Toxoplasma* infection. (A) Splenic CD4⁺ T cells were isolated from naive *Cxcr3*^{-/-} mice, and 5x10⁶ cells were adoptively transferred intravenously into *Cxcr3*^{-/-} recipients. Mice were orally challenged 24 hr later with 30 ME49 cysts and assessed for survival (n=5 mice per group). (B) Lamina propria leukocytes were harvested from infected WT and *Cxcr3*^{-/-} mice and cultured in the presence of Brefeldin-A for 6 hr. Cells were surface stained for CD11b, Ly6C/G (Gr-1), and Ly6G to identify inflammatory monocytes. Cells were then fixed, permeabilized, and intracellularly stained for IL-12 and TNF- α . Cytokine production by inflammatory monocytes was analyzed by flow cytometry. Shown are representative FACS plots of individual mice. (C) Lamina propria leukocytes were surface stained for CD11b, Ly6C/G (Gr-1), and Ly6G (1A8) to identify neutrophils. (D) Cells were then fixed, permeabilized, and intracellularly stained for TNF- α . Cytokine production by neutrophils was analyzed by flow cytometry. Shown are the mean \pm SEM of individual mice (n=5 mice per group; * p<0.05, ** p<0.01).

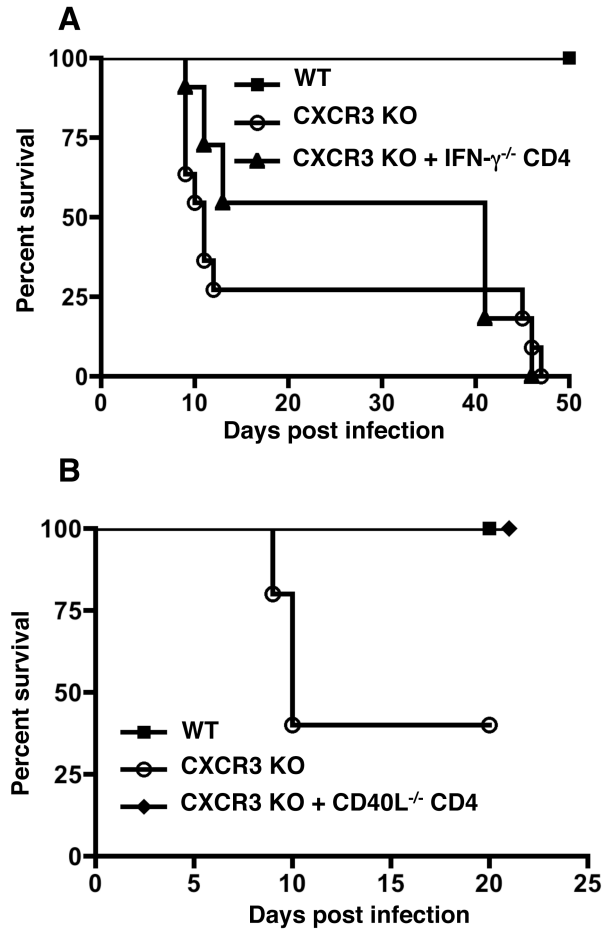


Figure 3.15. CD4-mediated rescue is dependent on IFN- γ but independent of CD40L. CD4⁺ T cells were isolated from *Ifn- γ ^{-/-}* (A) and *CD40L^{-/-}* (B) mice, adoptively transferred into *Cxcr3^{-/-}* mice that were subsequently challenged with *Toxoplasma* as described in the Figure 6 legend (WT: n=10; KO: n=11; *Ifn- γ ^{-/-}*: n=11; *CD40L^{-/-}*: n=5).

Discussion

Effective control of pathogens such as *T. gondii* requires the coordinated action of cells of innate and adaptive immunity. Orchestration of the response is governed by an underlying network of chemokines and chemokine receptor-expressing cells in both the hematopoietic and non-hematopoietic compartments. In this study, we demonstrate a central role for chemokine receptor CXCR3 in empowering Th1 trafficking to the small intestine, in turn enabling inflammatory monocyte activation and concomitant control of infection. While the importance of IFN- γ -secreting CD4⁺ T cells in resistance to *Toxoplasma* is well known to researchers in the field (28, 32, 33), and while the importance of anti-microbial inflammatory monocytes has recently become clear in the context of *Toxoplasma* and other infections (11), the present study is the first to reveal the functional link between CXCR3⁺ T-cell effectors, IFN- γ and inflammatory monocyte activation in tissues of the intestinal mucosa.

Although Th1 effectors depend upon CXCR3 to reach the site of infection, inflammatory monocytes require chemokine receptor CCR2 for optimal trafficking. In the latter case, inflammatory monocytes fail to exit the bone marrow in *Ccr2*^{-/-} mice, resulting in a decreased level of this population in the periphery, in turn resulting in inability to control *T. gondii* infection (11). Monocytes have also been suggested to promote the systemic dissemination of *T. gondii* to the brain (34). While brain parasite loads were not examined in this study, it is unlikely that altered parasite shuttling is a mechanism by which the knockout animals are succumbing to *Toxoplasma* because peripheral parasite loads were not affected by absence of CXCR3 (Figure 3.6B). Our

data argue that the basis for increased parasites specifically in the intestine is the result of defective regional control by inflammatory monocytes that lack CXCR3-dependent activation signals. Recent data indicate that inflammatory monocyte expression of CCR1 enables a response to IL-15-dependent CCL3-secreting innate lymphoid cells, resulting in CCR1-dependent recruitment to the intestinal mucosa of *Toxoplasma* infected mice (35). Taking these data and ours collectively, we propose that CXCR3, CCR2 and CCR1 act together as a control axis of innate and acquired immunity in intestinal immunity, ensuring coordinated recruitment of inflammatory monocytes and Th1 effectors to inflamed tissues.

It was recently demonstrated that NK cell-derived IFN- γ controls the differentiation of circulating monocytes into inflammatory dendritic cells during i. p. *T. gondii* infection, and is thus required for an optimal IL-12 response (36). In our model we did not see a dependence on CXCR3 for NK cell recruitment, as NK cells appeared to lose CXCR3-GFP expression over the course of infection (Figure 2.2), and production of NK cell IFN- γ was equivalent in WT and KO mice (Figure 3.10D-F). This difference may be attributable to the alternative routes of infection used, as our model incorporates the intestinal response, while the i.p. route bypasses the intestinal mucosa. Consistent with this idea, absence of CXCR3 did not affect the ability of mice to survive i. p. infection (Figure 3.5A).

While our study focuses on the early response to *Toxoplasma* infection in the intestinal mucosa, others have examined the role of CXCR3 and its ligands in additional tissues

and at different stages of infection. Antibody-mediated depletion of CXCL10, a major CXCR3 ligand, increases susceptibility and blocks influx and expansion of T cells in the liver and spleen that accompanies *T. gondii* infection (37). Additionally, a study of ocular toxoplasmosis revealed that T cells infiltrating the eye during infection express CXCR3 and produce IFN- γ . Depletion of CXCL10 in this model reduced the number of infiltrating T lymphocytes during chronic infection, resulting in increased parasite replication and ocular damage (38). Recently, CXCL10 was shown to impact CD8⁺ T-cell mobility in the brain of chronically infected mice, enhancing their ability to control the parasite by increasing contact with infected cells (39). Our results for the first time highlight the importance of CXCR3 and its impact on CCR2-dependent monocytes in the initial protective response to the parasite in the intestine.

It has been shown that TGF- β production by intestinal IEL protects against *T. gondii*-induced damage by down-modulating inflammation (40). While we did not examine intraepithelial lymphocytes (IEL) in this study, it is possible that CXCR3 expression could also affect trafficking and function of this cell type, thereby contributing to resistance in this model. Future studies will examine whether CXCR3-expressing IEL play a role in immunity to oral *T. gondii* infection.

CXCR3 has also been assessed for its role in immunity to other protozoan pathogens, including *Leishmania* and *Plasmodium*. Interestingly, the function of CXCR3 differs depending upon the parasite, the route of infection and the site examined. For example, *Cxcr3*^{-/-} mice exhibit impaired IFN- γ production and increased lesion

development during cutaneous *L. major* infection, but the knockout mice are not more susceptible to hepatic *L. donovani* infection (19, 41). Furthermore, CXCR3 and its chemokines promote cerebral inflammation and mortality during experimental malaria infection (20, 42). Overall, CXCR3 and its chemokine ligands function as double-edged swords, inasmuch as they make an important contribution to protective immunity, but when dysregulated they are the cause of deleterious immunopathology.

In addition to its role in cell recruitment, CXCR3 has recently been suggested to be important for priming CD4⁺ T cells in the lymph node to become Th1 cells by promoting long-lasting interactions between T cells and CXCL10-expressing dendritic cells. In the absence of CXCR3, T cells fail to fully differentiate into IFN- γ -producing cells and are defective during subsequent lymphocytic choriomeningitis virus (LCMV) infection (43). We found no evidence for defective Th1 responses in secondary lymphoid organs during *Toxoplasma* infection of *Cxcr3*^{-/-} mice, a result that is supported by similar findings during *L. major* infection (19). However, our results are consistent with impaired recruitment of Th1 cells in the absence of CXCR3, as lamina propria CD4⁺ T cells from *Cxcr3*^{-/-} mice exhibited defective IFN- γ production. We conclude that the function of CXCR3 in promoting Th1 differentiation versus, or in addition to, homing to inflammatory sites is likely to be a context-dependent phenomenon.

Although we found no differences in T-cell activation in CXCR3 negative T cells of reporter mice or in CXCR3 KO animals, as measured by expression of CD25, CD69

and CD44 (data not shown), we observed a consistent decrease in CD27 expression amongst CXCR3-negative T cells of reporter mice. Overall levels of CD27 expression were also lower in T cells from *Cxcr3*^{-/-} mice (data not shown). CD27 is a member of the TNF receptor super family that has been implicated as a T-cell costimulatory molecule (44). CD27 expression is thought to characterize naïve or memory T cells, whereas loss of CD27 represents terminal differentiation (45). Accordingly, it is possible that in addition to controlling T-cell recruitment to sites of infection, *Cxcr3*^{-/-} T cells may undergo terminal differentiation and, possibly, premature Th1 effector death in tissues prior to mediating IFN- γ -dependent inflammatory monocyte activation. This is consistent with studies showing that CD27^{low} cells are more susceptible to apoptosis and that accumulation of influenza-specific T lymphocytes was impaired in the lungs of CD27^{-/-} mice during infection (44, 46). In our study, CD27 expression was not rescued by adoptive transfer of WT CD4⁺ T cells (data not shown), which may suggest that increasing the T-cell pool allows the system to cross a certain threshold of T-cell levels in order to activate inflammatory monocytes without reversing CD27 expression.

In the absence of CXCR3, we found that lamina propria neutrophil levels were increased during infection, as was their activation status as measured by TNF- α expression. Furthermore, adoptive transfer of WT CD4⁺ T lymphocytes into the KO strain reversed increased levels of PMN as well as their production of TNF- α . Because this inflammatory cytokine has been linked to intestinal damage during *Toxoplasma* infection (47), it seems likely that CXCR3-dependent effects on neutrophils are likely

to be secondary to loss of inflammatory monocyte function. In this scenario, defects in monocyte-mediated parasite killing would result in damage to the intestinal mucosa. Translocation of luminal gut flora, known to contribute to emergence of parasite-induced intestinal lesions (48-50), would in turn be expected to result in local neutrophil recruitment. Indeed, based on neutrophil depletion studies it has been suggested that these cells mediate damage to the intestinal mucosa during *T. gondii* infection (27). The cellular and molecular basis for this effect is not at present known, but both the IL-17/IL-23 axis and CXCL8 have been shown to promote neutrophil accumulation in infected tissues suggesting involvement of one or both of these mediators (51-53).

The results of this study extend our understanding of immunity in the intestinal mucosa, which has become increasingly important as inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, become more common in developed regions of the world. In this regard, abnormally high levels of CXCR3 are associated with dysregulated intestinal responses in human IBD patients, underscoring the potential hazards of unbalanced inflammatory responses (54-56). While CXCR3 can be pathogenic by recruiting effector cells to otherwise healthy tissue, as in IBD or cerebral malaria, we show here that CXCR3-expressing T cells play an essential protective role in host defense by enabling defense against pathogenic organisms. Antibodies against CXCL10 have been suggested as potential therapeutic agents against IBD (57, 58). The results from this study, however, highlight the possible

harm of inhibiting CXCR3-expressing cells into sites of inflammation during infection with an enteric pathogen.

As the first study to demonstrate a protective role for CXCR3⁺CD4⁺ T cells in the intestinal immune response, we have shown here that failure to appropriately recruit these T cells results in impaired inflammatory monocyte activation, accumulation of intestinal parasites, and subsequent recruitment of potentially pathogenic TNF- α -secreting neutrophils. Our results reveal CXCR3 as a critical chemokine receptor of the adaptive immune system that ensures appropriate placement of T cells in inflamed tissue, enabling inflammatory monocytes of innate immunity to acquire effector functions and mediate effective host defense.

Materials and Methods

Mice and Infections

Female Swiss Webster mice (6-8 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME). *Cxcr3*^{-/-} and *Cxcr3* eGFP knock-in reporter mice (21) were established as breeding colonies in the Transgenic Mouse Facility at the Cornell University College of Veterinary Medicine. The CXCR3 internal ribosomal entry site bicistronic eGFP reporter (CIBER) mice were generated as described (21). This strain possesses a functional CXCR3 receptor, and all CXCR3 positive cells also express intracellular eGFP. Mouse infections were initiated by oral inoculation of cysts of the type II *T. gondii* ME49 strain. Cysts were isolated from chronically infected Swiss Webster mice by homogenization of whole brain in sterile PBS. Unless stated otherwise, mice were infected at 8-12 weeks of age with 30 cysts.

Tissue staining and immunofluorescence microscopy

Intestines were excised, flushed with 10% neutral-buffered formaldehyde, and embedded in paraffin for sectioning. Sections were stained for hematoxylin and eosin for assessment of pathological changes. Sections were also stained for parasite antigen by immunohistochemistry at the Cornell Animal Health Diagnostic Center. Frozen sections were obtained by embedding 1 cm lengths of intestine in OCT. Sections of 6-8µm were cut on a cryostat, fixed in ice-cold acetone, and blocked with PBS containing 2X casein and goat serum. To examine T-cell infiltration, sections were incubated with rat anti-CD4 antibody (GK1.5) (ATCC, Manassas, VA) or rat IgG at 4°C overnight followed by goat anti-rat Alexa-647 secondary antibody (Life

Technologies, Grand Island, NY). Sections were then mounted in DAPI-Prolong antifade (Life Technologies) and imaged by confocal microscopy. ImageJ software was used to analyze fluorescence of independent channels.

Cell isolation

Spleens and mesenteric lymph nodes were excised, crushed between sterile slides, and passed through a 70- μ m filter (BD, Franklin Lakes, NJ). Red blood cells from splenocyte suspensions were lysed with ACK lysis buffer (Life Technologies). For LP leukocyte isolation, the small intestine was removed, cleaned of mesentery, flushed with sterile PBS, and cleared of Peyer's patches. The intestine was opened longitudinally and the mucosal layer was scraped with a blunt scalpel to remove epithelial cells. The tissue was cut into 5 mm sections and vigorously washed with Dulbecco's modified Eagle's media (Cellgro, Manassas, VA) and 5 mM EDTA (Life Technologies). Cells were liberated from the intestinal tissue by digestion with 10mg/ml collagenase (Sigma, St. Louis, MO) at 37°C and subsequently passed through a 70- μ m filter.

Cytokine measurement

Secretion of IFN- γ , TNF- α , and IL-10 was assayed by ELISA (eBioscience, San Diego, CA) following manufacturer's instructions in the presence of soluble tachyzoite antigen (STAg) prepared as previously described (59). IL-12p40 was quantitated using an in-house ELISA (60). For ileum biopsy cultures, 1 cm intestinal sections were flushed with PBS, opened longitudinally, and cultured overnight in

complete Dulbecco's modified Eagle's media supplemented with 10% bovine growth serum (Hyclone), 0.05 mM β -mercaptoethanol (Sigma), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10,000 U/ml penicillin, 10,000 mg/ml streptomycin, and 30 mM HEPES (reagents from Life Technologies). Supernatants were collected and assayed for cytokine by ELISA.

Measurement of mRNA by quantitative PCR

RNA was isolated from MLN and intestinal tissue from mice over a time course of infection. Tissue was initially disrupted with a tissue homogenizer and subjected to RNA isolation following manufacturer's instructions (Tissue RNA Kit, Omega Biotek, Norcross, GA). RNA was converted to cDNA (Quanta Biosciences, Gaithersburg, MD) and assayed for gene expression by SYBR green technology (Quanta Biosciences). Primers were designed to span exons by Integrated DNA Technologies. GAPDH was used as a housekeeping gene. Gene expression from each timepoint was normalized to uninfected control samples.

Flow cytometry

Single cell suspensions were pelleted and resuspended with primary antibodies (BioLegend, San Diego, CA: anti-CD4 PerCP, anti-CD8a APC-Cy7, anti-CD45 Alexa-488, anti-CD11b APC-Cy7, anti-CD11b APC, anti-Ly6G FITC; eBioscience, San Diego, CA: anti-CD25 PE, anti-CD45 APC or FITC, anti-CD69 PE, anti-Ly6C/G APC; BD Biosciences, San Jose, CA: anti-Ly6G PE-Cy7, anti-Ly6C V420, anti-CD45 PE, anti-Ly6C/G PerCP, anti-CD44 APC) in ice-cold FACS buffer (1% bovine serum

albumin/0.01% NaN₃ in PBS) for 30 min. For IFN- γ staining, cells were incubated for 6 hrs with Brefeldin-A (eBioscience; 10 μ g/ml), PMA (Sigma; 10ng/ml), and ionomycin (Sigma; 1 μ g/ml), then fixed with 4% paraformaldehyde and subsequently incubated with primary antibodies resuspended in the FoxP3/transcription factor buffer staining set (eBioscience). For IL-12 and TNF- α staining, cells were incubated for 6 hrs with Brefeldin-A only (eBioscience). Intracellular staining experiments used 10⁶ cells. Antibodies used for intracellular staining included anti-IFN- γ PE-Cy7, anti-TNF- α PE-Cy7 (Biolegend); anti-IL-12 PE, anti-TNF- α APC (BD Biosciences), and anti-*Toxoplasma* p30 (Argene, Shirley, NY). Cell fluorescence was measured using a FACS Canto (BD Biosciences). Data was analyzed using FlowJo software (FlowJo, Ashland, OR).

Quantitative PCR for parasite burden

DNA was isolated from whole intestinal tissue using a Tissue DNA kit following manufacturer's instructions (Omega Biotek, Norcross, GA). DNA was amplified by quantitative PCR as described previously using primers against the *T. gondii* B1 gene and the host argininosuccinate lyase (ASL) gene (61). Ten-fold serial dilutions of genome copy standard curves were created using known quantities of host (splenocytes) and parasite (tachyzoites) cells based on DNA quantity, Avogadro's number, and genome size. To quantify parasite burden, the generated values for host and parasite genome copies from the DNA preparations were expressed as a ratio of parasite (B1) to host (ASL) genomes.

Adoptive transfer

Splenocytes from naïve mice were harvested and subjected to CD4 positive selection by magnetic bead sorting following manufacturer's instructions (Stem Cell Technologies, Vancouver, British Columbia). Cells were purified to ~90-95% purity and were transferred by intravenous retro-orbital injection into *Cxcr3*^{-/-} recipients at 5x10⁶ CD4⁺ cells per mouse. Twenty-four hours post transfer, mice were challenged with 30 cysts of the *T. gondii* ME49 strain. In some experiments mice were left to assess survival following cell transfer. In other experiments intestinal tissue was collected at day 9 post-infection for intracellular cytokine analysis.

Pathology scoring

Swiss rolls of the intestines were histopathologically scored by an investigator that was double-blinded to sample identity. Intestines were scored on an ascending 0-4 scale as previously described (50, 62). Briefly, scores of 0 were normal, scores of 1 indicated mild focal lesions, scores of 2 indicated moderate focal lesions, scores of 3 indicated moderate multifocal lesions, and scores of 4 indicated severe multifocal lesions. Histopathological features scored included: inflammation of the intestinal submucosa (lamina propria), inflammation extending throughout all histological layers of the intestine (transmural inflammation), sloughing of intestinal epithelium, intestinal villus fusion and blunting, and necrosis of villi. Sections of complete small intestines from 13 KO and 14 WT infected mice were scored. The data are plotted as mean scores of individual mice.

Statistics

Differences between groups were analyzed using student's *t*-test. Differences between 3 or more groups were analyzed using one-way Anova with Newman-Keuls post-test. Survival comparisons were made using a Kaplan-Meier curve (Logrank test). P-values less than 0.05 are considered significant and are designated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. Pathology scores were analyzed using the Mann-Whitney *t*-test.

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References

1. Denkers, E. Y., and R. T. Gazzinelli. 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin. Microbiol. Rev.* 11: 569–588.
2. Dupont, C. D., D. A. Christian, and C. A. Hunter. 2012. Immune response and immunopathology during toxoplasmosis. *Semin Immunopathol* 34: 793–813.
3. Scanga, C. A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E. Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. *J. Immunol.* 168: 5997–6001.
4. Yarovinsky, F., D. Zhang, J. F. Andeuren, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh, and A. Sher. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 308: 1626–1629.
5. Koblansky, A. A., D. Jankovic, H. Oh, S. Hieny, W. Sungnak, R. Mathur, M. S. Hayden, S. Akira, A. Sher, and S. Ghosh. 2012. Recognition of Profilin by Toll-like Receptor 12 Is Critical for Host Resistance to *Toxoplasma gondii*. *Immunity*.
6. Andrade, W. A., M. D. C. Souza, E. Ramos-Martinez, K. Nagpal, M. S. Dutra, M. B. Melo, D. C. Bartholomeu, S. Ghosh, D. T. Golenbock, and R. T. Gazzinelli. 2013. Combined action of nucleic acid-sensing Toll-like receptors and TLR11/TLR12 heterodimers imparts resistance to *Toxoplasma gondii* in mice. *Cell Host Microbe* 13: 42–53.
7. Sukhumavasi, W., C. E. Egan, A. L. Warren, G. A. Taylor, B. A. Fox, D. J. Bzik, and E. Y. Denkers. 2008. TLR adaptor MyD88 is essential for pathogen control during oral *Toxoplasma gondii* infection but not adaptive immunity induced by a vaccine strain of the parasite. *The Journal of Immunology* 181: 3464–3473.
8. Zhao, Y. O., A. Khaminets, J. P. Hunn, and J. C. Howard. 2009. Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFN γ -inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. *PLoS Pathog* 5: e1000288.
9. Taylor, G. A. 2007. IRG proteins: key mediators of interferon-regulated host resistance to intracellular pathogens. *Cell. Microbiol.* 9: 1099–1107.
10. Khaminets, A., J. P. Hunn, S. Könen-Waisman, Y. O. Zhao, D. Preukschat, J. Coers, J. P. Boyle, Y.-C. Ong, J. C. Boothroyd, G. Reichmann, and J. C. Howard. 2010. Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell. Microbiol.* 12: 939–961.

11. Dunay, I. R., R. A. Damatta, B. Fux, R. Presti, S. Greco, M. Colonna, and L. D. Sibley. 2008. Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. *Immunity* 29: 306–317.
12. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71–82.
13. Shi, C., T. M. Hohl, I. Leiner, M. J. Equinda, X. Fan, and E. G. Pamer. 2011. Ly6G⁺ neutrophils are dispensable for defense against systemic *Listeria monocytogenes* infection. *The Journal of Immunology* 187: 5293–5298.
14. Serbina, N. V., T. P. Salazar-Mather, C. A. Biron, W. A. Kuziel, and E. G. Pamer. 2003. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 19: 59–70.
15. Peters, W., H. M. Scott, H. F. Chambers, J. L. Flynn, I. F. Charo, and J. D. Ernst. 2001. Chemokine receptor 2 serves an early and essential role in resistance to *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 98: 7958–7963.
16. Peters, W., J. G. Cyster, M. Mack, D. Schlöndorff, A. J. Wolf, J. D. Ernst, and I. F. Charo. 2004. CCR2-dependent trafficking of F4/80^{dim} macrophages and CD11c^{dim}/intermediate dendritic cells is crucial for T cell recruitment to lungs infected with *Mycobacterium tuberculosis*. *J. Immunol.* 172: 7647–7653.
17. Ling, Y. M., M. H. Shaw, C. Ayala, I. Coppens, G. A. Taylor, D. J. P. Ferguson, and G. S. Yap. 2006. Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. *J. Exp. Med.* 203: 2063–2071.
18. Groom, J. R., and A. D. Luster. 2011. CXCR3 in T cell function. *Experimental Cell Research* 317: 620–631.
19. Rosas, L. E., J. Barbi, B. Lu, Y. Fujiwara, C. Gerard, V. M. Sanders, and A. R. Satoskar. 2005. CXCR3^{-/-} mice mount an efficient Th1 response but fail to control *Leishmania major* infection. *Eur. J. Immunol.* 35: 515–523.
20. Campanella, G. S. V., A. M. Tager, J. K. El Khoury, S. Y. Thomas, T. A. Abrazinski, L. A. Manice, R. A. Colvin, and A. D. Luster. 2008. Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. *Proc. Natl. Acad. Sci. U.S.A.* 105: 4814–4819.
21. Oghumu, S., R. Dong, S. Varikuti, T. Shawler, T. Kampfrath, C. A. Terrazas, C. Lezama-Davila, B. M. M. Ahmer, C. C. Whitacre, S. Rajagopalan, R. Locksley, A. H. Sharpe, and A. R. Satoskar. 2013. Distinct populations of innate CD8⁺ T cells revealed in a CXCR3 reporter mouse. *The Journal of Immunology* 190: 2229–2240.

22. Qin, S., J. B. Rottman, P. Myers, N. Kassam, M. Weinblatt, M. Loetscher, A. E. Koch, B. Moser, and C. R. Mackay. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* 101: 746–754.
23. Sher, A., I. P. Oswald, S. Hieny, and R. T. Gazzinelli. 1993. *Toxoplasma gondii* induces a T-independent IFN-gamma response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor-alpha. *J. Immunol.* 150: 3982–3989.
24. Heimesaat, M. M., A. Fischer, H.-K. Jahn, J. Niebergall, M. Freudenberg, M. Blaut, O. Liesenfeld, R. R. Schumann, U. B. Göbel, and S. Bereswill. 2007. Exacerbation of murine ileitis by Toll-like receptor 4 mediated sensing of lipopolysaccharide from commensal *Escherichia coli*. *Gut* 56: 941–948.
25. Waddell, A., R. Ahrens, Y.-T. Tsai, J. D. Sherrill, L. A. Denson, K. A. Steinbrecher, and S. P. Hogan. 2013. Intestinal CCL11 and eosinophilic inflammation is regulated by myeloid cell-specific RelA/p65 in mice. *The Journal of Immunology* 190: 4773–4785.
26. Ueno, N., M. Fujiya, S. Segawa, T. Nata, K. Moriichi, H. Tanabe, Y. Mizukami, N. Kobayashi, K. Ito, and Y. Kohgo. 2011. Heat-killed body of *Lactobacillus brevis* SBC8803 ameliorates intestinal injury in a murine model of colitis by enhancing the intestinal barrier function. *Inflamm. Bowel Dis.* 17: 2235–2250.
27. Dunay, I. R., A. Fuchs, and L. D. Sibley. 2010. Inflammatory monocytes but not neutrophils are necessary to control infection with *Toxoplasma gondii* in mice. *Infection and Immunity* 78: 1564–1570.
28. Scharton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, S. Hieny, R. T. Gazzinelli, and A. Sher. 1996. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to *Toxoplasma gondii* while failing to control acute infection. *J. Immunol.* 157: 4045–4054.
29. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kühn, W. Müller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4⁺ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J. Immunol.* 157: 798–805.
30. Mashayekhi, M., M. M. Sandau, I. R. Dunay, E. M. Frickel, A. Khan, R. S. Goldszmid, A. Sher, H. L. Ploegh, T. L. Murphy, L. D. Sibley, and K. M. Murphy. 2011. CD8 α (+) dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites. *Immunity* 35: 249–259.

31. Li, W., D. Buzoni-Gatel, H. Debbabi, M. S. Hu, F. J. D. Mennechet, B. G. Durell, R. J. Noelle, and L. H. Kasper. 2002. CD40/CD154 ligation is required for the development of acute ileitis following oral infection with an intracellular pathogen in mice. *Gastroenterology* 122: 762–773.
32. Gazzinelli, R. T., F. T. Hakim, S. Hieny, G. M. Shearer, and A. Sher. 1991. Synergistic role of CD4⁺ and CD8⁺ T lymphocytes in IFN- γ production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J. Immunol.* 146: 286–292.
33. Suzuki, Y., M. A. Orellana, R. D. Schreiber, and J. S. Remington. 1988. Interferon- γ : the major mediator of resistance against *Toxoplasma gondii*. *Science* 240: 516–518.
34. Courret, N., S. Darche, P. Sonigo, G. Milon, D. Buzoni-Gatel, and I. Tardieux. 2006. CD11c- and CD11b-expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain. *Blood* 107: 309–316.
35. Schulthess, J., B. Meresse, E. Ramiro-Puig, N. Montcuquet, S. Darche, B. Bègue, F. Ruemmele, C. Combadière, J. P. Di Santo, D. Buzoni-Gatel, and N. Cerf-Bensussan. 2012. Interleukin-15-dependent NKp46⁺ innate lymphoid cells control intestinal inflammation by recruiting inflammatory monocytes. *Immunity* 37: 108–121.
36. Goldszmid, R. S., P. Caspar, A. Rivollier, S. White, A. Dzutsev, S. Hieny, B. Kelsall, G. Trinchieri, and A. Sher. 2012. NK cell-derived interferon- γ orchestrates cellular dynamics and the differentiation of monocytes into dendritic cells at the site of infection. *Immunity* 36: 1047–1059.
37. Khan, I. A., J. A. MacLean, F. S. Lee, L. Casciotti, E. DeHaan, J. D. Schwartzman, and A. D. Luster. 2000. IP-10 is critical for effector T cell trafficking and host survival in *Toxoplasma gondii* infection. *Immunity* 12: 483–494.
38. Norose, K., A. Kikumura, A. D. Luster, C. A. Hunter, and T. H. Harris. 2011. CXCL10 is required to maintain T-cell populations and to control parasite replication during chronic ocular toxoplasmosis. *Invest. Ophthalmol. Vis. Sci.* 52: 389–398.
39. Harris, T. H., E. J. Banigan, D. A. Christian, C. Konradt, E. D. Tait Wojno, K. Norose, E. H. Wilson, B. John, W. Weninger, A. D. Luster, A. J. Liu, and C. A. Hunter. 2012. Generalized Lévy walks and the role of chemokines in migration of effector CD8⁺ T cells. *Nature* 486: 545–548.
40. Buzoni-Gatel, D., H. Debbabi, F. J. Mennechet, V. Martin, A. C. Lepage, J. D. Schwartzman, and L. H. Kasper. 2001. Murine ileitis after intracellular parasite infection is controlled by TGF- β -producing intraepithelial lymphocytes.

Gastroenterology 120: 914–924.

41. Barbi, J., S. Oghumu, L. E. Rosas, T. Carlson, B. Lu, C. Gerard, C. M. Lezama-Davila, and A. R. Satoskar. 2007. Lack of CXCR3 delays the development of hepatic inflammation but does not impair resistance to *Leishmania donovani*. *J. Infect. Dis.* 195: 1713–1717.
42. Nie, C. Q., N. J. Bernard, M. U. Norman, F. H. Amante, R. J. Lundie, B. S. Crabb, W. R. Heath, C. R. Engwerda, M. J. Hickey, L. Schofield, and D. S. Hansen. 2009. IP-10-Mediated T Cell Homing Promotes Cerebral Inflammation over Splenic Immunity to Malaria Infection. *PLoS Pathog* 5: e1000369.
43. Groom, J. R., J. Richmond, T. T. Murooka, E. W. Sorensen, J. H. Sung, K. Bankert, U. H. von Andrian, J. J. Moon, T. R. Mempel, and A. D. Luster. 2012. CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4⁺ T helper 1 cell differentiation. *Immunity* 37: 1091–1103.
44. Hendriks, J., L. A. Gravestien, K. Tesselaar, R. A. van Lier, T. N. Schumacher, and J. Borst. 2000. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat. Immunol.* 1: 433–440.
45. Hamann, D., P. A. Baars, M. H. Rep, B. Hooibrink, S. R. Kerkhof-Garde, M. R. Klein, and R. A. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8⁺ T cells. *J. Exp. Med.* 186: 1407–1418.
46. Kapina, M. A., G. S. Shepelkova, V. V. Mischenko, P. Sayles, P. Bogacheva, G. Winslow, A. S. Apt, and I. V. Lyadova. 2007. CD27^{low} CD4 T lymphocytes that accumulate in the mouse lungs during mycobacterial infection differentiate from CD27^{high} precursors in situ, produce IFN-gamma, and protect the host against tuberculosis infection. *J. Immunol.* 178: 976–985.
47. Liesenfeld, O., H. Kang, D. Park, T. A. Nguyen, C. V. Parkhe, H. Watanabe, T. Abo, A. Sher, J. S. Remington, and Y. Suzuki. 1999. TNF-alpha, nitric oxide and IFN-gamma are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible mice infected perorally with *Toxoplasma gondii*. *Parasite Immunol.* 21: 365–376.
48. Heimesaat, M. M., S. Bereswill, A. Fischer, D. Fuchs, D. Struck, J. Niebergall, H.-K. Jahn, I. R. Dunay, A. Moter, D. M. Gescher, R. R. Schumann, U. B. Göbel, and O. Liesenfeld. 2006. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. *J. Immunol.* 177: 8785–8795.
49. Craven, M., C. E. Egan, S. E. Dowd, S. P. McDonough, B. Dogan, E. Y. Denkers, D. Bowman, E. J. Scherl, and K. W. Simpson. 2012. Inflammation drives dysbiosis

and bacterial invasion in murine models of ileal Crohn's disease. *PLoS ONE* 7: e41594.

50. Egan, C. E., M. D. Craven, J. Leng, M. Mack, K. W. Simpson, and E. Y. Denkers. 2009. CCR2-dependent intraepithelial lymphocytes mediate inflammatory gut pathology during *Toxoplasma gondii* infection. *Mucosal Immunol* 2: 527–535.

51. Del Rio, L., S. Bennouna, J. Salinas, and E. Y. Denkers. 2001. CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during *Toxoplasma gondii* infection. *J. Immunol.* 167: 6503–6509.

52. Wu, Q., R. J. Martin, J. G. Rino, R. Breed, R. M. Torres, and H. W. Chu. 2007. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory *Mycoplasma pneumoniae* infection. *Microbes Infect.* 9: 78–86.

53. Murphy, P. M. 1997. Neutrophil receptors for interleukin-8 and related CXC chemokines. *Semin. Hematol.* 34: 311–318.

54. Papadakis, K. A., J. Prehn, D. Zhu, C. Landers, J. Gaiennie, P. R. Fleshner, and S. R. Targan. 2004. Expression and regulation of the chemokine receptor CXCR3 on lymphocytes from normal and inflammatory bowel disease mucosa. *Inflamm. Bowel Dis.* 10: 778–788.

55. Yuan, Y. H., T. ten Hove, F. O. The, J. F. Slors, S. J. van Deventer, and A. A. te Velde. 2001. Chemokine receptor CXCR3 expression in inflammatory bowel disease. *Inflamm. Bowel Dis.* 7: 281–286.

56. Schroepf, S., R. Kappler, S. Brand, C. Prell, P. Lohse, J. Glas, E. Hoster, J. Helmbrecht, A. Ballauff, M. Berger, D. von Schweinitz, S. Koletzko, and M. Lacher. 2010. Strong overexpression of CXCR3 axis components in childhood inflammatory bowel disease. *Inflamm. Bowel Dis.* 16: 1882–1890.

57. Nishimura, M., Y. Kuboi, K. Muramoto, T. Kawano, and T. Imai. 2009. Chemokines as novel therapeutic targets for inflammatory bowel disease. *Ann. N. Y. Acad. Sci.* 1173: 350–356.

58. Singh, U. P., C. Venkataraman, R. Singh, and J. W. Lillard. 2007. CXCR3 axis: role in inflammatory bowel disease and its therapeutic implication. *Endocr Metab Immune Disord Drug Targets* 7: 111–123.

59. Denkers, E. Y., R. T. Gazzinelli, S. Hieny, P. Caspar, and A. Sher. 1993. Bone marrow macrophages process exogenous *Toxoplasma gondii* polypeptides for recognition by parasite-specific cytolytic T lymphocytes. *J. Immunol.* 150: 517–526.

60. Butcher, B. A., L. Kim, P. F. Johnson, and E. Y. Denkers. 2001. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-kappa B. *J. Immunol.* 167: 2193–2201.
61. Butcher, B. A., B. A. Fox, L. M. Rommereim, S. G. Kim, K. J. Maurer, F. Yarovinsky, D. R. Herbert, D. J. Bzik, and E. Y. Denkers. 2011. *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog* 7: e1002236.
62. Johnson, L. L., P. Lanthier, J. Hoffman, and W. Chen. 2004. Vaccination protects B cell-deficient mice against an oral challenge with mildly virulent *Toxoplasma gondii*. *Vaccine* 22: 4054–4061.

CHAPTER 4

β -Catenin Signaling Drives Differentiation and Proinflammatory Function of IRF8-Dependent Dendritic Cells*

*Based on: Cohen, SB, Pepper, M, Shah, S, Yap, GS, Jiang, A, Clausen, BE, Denkers, EY. “ β -catenin Signaling Drives Differentiation and Proinflammatory Function of IRF8-Dependent Dendritic Cells.” In review.

Abstract

Signaling through the Wnt/ β -catenin pathway has recently been tied to emergence and function of tolerogenic dendritic cells (DC). Here we demonstrate a new role for β -catenin in directing development of DC subsets through control of cell survival and interferon regulatory factor (IRF) 8 activation. DC from mice overexpressing β -catenin displayed increased proliferation and IRF8 expression, leading to expansion of IRF8-dependent CD8 α^+ , plasmacytoid, and peripheral CD103 $^+$ DC subsets. As a result of increased IRF8 expression, β -catenin stabilized CD8 α^+ DC secreted elevated levels of IL-12 upon in vitro microbial stimulation, and infection of mutant mice with the protozoan parasite *Toxoplasma gondii* led to increased susceptibility associated with hypersecretion of IL-12 and Th1-related cytokines. These results reveal a novel function for β -catenin in programming DC identity towards pro-inflammatory subsets in dependence on IRF8.

Introduction

Dendritic cells (DC) critically bridge innate and adaptive immunity through their exquisite capacity to drive antigen-specific T cell activation and effector subset differentiation. Furthermore, DC are central players in determining tolerance versus immunity during inflammation and infection (1). DC are a heterogeneous population of cells with varying surface markers and transcription factor requirements. All DC originate from a common bone marrow progenitor, but these cells subsequently differentiate into distinct subsets, including monocyte-derived DC (moDC), conventional DC (cDC), and plasmacytoid DC (pDC). Many elegant studies have identified phenotypic and functional differences amongst these subsets, but identifying factors determining control points of DC subset generation is a continuing focus of intense interest. Several key cytokines and transcription factors have been implicated in controlling DC developmental pathways (2), and recent gene mapping studies have begun to elucidate the order in which these factors become expressed (2-5). For example, transcription factor Batf3 is involved in generation of splenic CD8 α^+ DC, while IRF4 is important in differentiation of CD11b $^+$ CD103 $^+$ DC in the intestinal lamina propria (6, 7). Recently, Zbtb46 was identified as a global transcription factor necessary for generation of cDC (4, 8). Nevertheless, a thorough understanding of the mechanisms of DC differentiation and the signals that direct branch points leading to distinct subsets remains incomplete.

β -catenin is the primary mediator of the Wnt signaling pathway and is critical for numerous cellular functions, including hematopoietic cell fate determination and

proliferation (9). Cytosolic β -catenin levels are normally maintained at low levels through continual phosphorylation by the serine threonine kinases glycogen synthase kinase (GSK)-3 β and casein kinase (CK) I- α , which cooperate to promote its ubiquitination and proteosomal degradation. Activating Wnt ligands trigger disassembly of the complex that coordinates these kinases, leaving β -catenin unphosphorylated, in turn enabling nuclear translocation for transcriptional activity in association with T cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors (10). While normally associated with embryonic development and tumorigenesis (11), β -catenin is increasingly being recognized for its role in immunity (12). This is particularly the case for DC, where β -catenin signaling was first implicated in cluster disruption-mediated maturation towards a tolerogenic phenotype during in vitro culture (13). More recently, β -catenin was found to be involved in the generation or maintenance of tolerogenic DC subsets in the intestinal mucosa (1).

Here, we provide surprising new insight into the role of β -catenin in DC function by employing transgenic mice with a CD11c-specific deletion in the third exon of the β -catenin gene (14). The exon 3 fragment encodes the β -catenin amino acid sequence that is targeted for GSK-3 β -mediated serine threonine phosphorylation and subsequent degradation. Removal of this region through Cre-lox mediated excision therefore results in phosphorylation-resistant and constitutively active β -catenin (15). We made the unexpected discovery that β -catenin stabilization in DC results in selective expansion of steady-state levels of splenic CD8 α^+ DC, pDC, and peripheral CD103 $^+$

DC. These DC subsets share in common a dependence on IRF8 for their differentiation, and in accordance with this observation, we show that constitutive β -catenin signaling increases IRF8 expression by these DC subsets. Furthermore, β -catenin stabilization leads to enhanced proliferation and resistance to apoptosis by $CD8\alpha^+$ DC. We employed infection with the intracellular pathogen *Toxoplasma gondii* as a model to determine the in vivo consequences of DC-specific β -catenin stabilization. Control of *T. gondii* is well known to require robust Th1 activation (16, 17), and IL-12 produced by splenic $CD8\alpha^+$ DC has recently been shown to play an essential role in this response (18). Nevertheless, production of IL-12 and other proinflammatory cytokines is lethal if not modulated by endogenous mediators (19, 20). Indeed, exon 3-deleted mice were highly susceptible to infection, and this was associated with elevated levels of $CD8\alpha^+$ DC IL-12 secretion and increased Th1 IFN- γ production. Our results uncover a new role for β -catenin in controlling IRF8 expression in DC, thereby revealing this transcription factor as a key player regulating IRF8-driven DC differentiation and proinflammatory function.

Results

β -catenin stabilization expands splenic CD8 α^+ and plasmacytoid dendritic cell populations

To determine the role of β -catenin signaling in dendritic cell differentiation and function, mice floxed for exon 3 of the β -catenin gene were crossed with CD11c-cre animals, resulting in Cre-positive progeny whose CD11c $^+$ cells possessed an exon 3-deleted β -catenin form resistant to phosphorylation-induced degradation (14, 15). Flow cytometric analysis of CD11c $^+$ splenic DC from Cre-positive offspring (Ex3^{DC-/-} mice) demonstrated high β -catenin expression levels compared to Cre-negative littermate controls (Ex3^{fl/fl} mice) (Figure 4.1A), indicating accumulation of β -catenin protein upon exon 3 deletion. This was confirmed to be specific to CD11c $^+$ cells, as CD4 $^+$ T cells from Ex3^{DC-/-} mice did not display upregulation of β -catenin compared to CD4 $^+$ T cells from Ex3^{fl/fl} mice (Figure 4.1B). Furthermore, upon cytoplasmic and nuclear fractionation, Ex3^{DC-/-} bone marrow-derived DC (BMDC) were found to be enriched for a truncated form of nuclear β -catenin compared to Ex3^{fl/fl} DC, confirming enhanced nuclear translocation of protein (Figure 4.1C). Thus, CD11c-directed exon 3 deletion results in β -catenin accumulation and nuclear translocation in dendritic cells.

We next examined the outcome of β -catenin stabilization on tissue resident DC. We first observed that the percentage and total number of DC (CD11c $^+$ MHCII $^+$ cells) were unaffected in Ex3^{DC-/-} mice (Figure 4.2A-C). However, further analysis revealed a striking expansion of the CD8 α^+ DC subset and a concomitant decrease in CD11b $^+$

DC (Figure 4.2D-H). Furthermore, plasmacytoid DC (pDC), as defined by expression of B220 and PDCA-1, were also expanded in the spleens of $Ex3^{DC-/-}$ mice (Figure 4.2I-K). These collective data suggest that β -catenin exerts major effects on the generation or stability of splenic tissue resident DC populations. Therefore, employing wild-type (WT) mice as a source of cells, we assessed β -catenin levels in $CD8\alpha^+$ and pDC splenic populations. We found that these subsets displayed strikingly higher β -catenin levels compared to $CD8\alpha^-$ and PDCA-1 $^-$ populations (Figure 4.2L and M). These data lead us to conclude that β -catenin stabilization in DC promotes the expansion of $CD8\alpha^+$ DC and pDC.

The $CD8\alpha^+$ DC subset is developmentally and functionally related to CD103-expressing DC found in non-lymphoid peripheral tissues, such as the intestine and the lung (15, 21). Further examination of $Ex3^{DC-/-}$ mice revealed a striking expansion of intestinal $CD103^+CD11b^-$ cells but not $CD103^+CD11b^+$ cells (Figure 4.3A-C). Additionally, lung resident $CD103^+CD11b^-$ DC were also expanded in $Ex3^{DC-/-}$ mice compared to $Ex3^{fl/fl}$ littermate controls (Figure 4.3D and E). These results demonstrate the capacity of β -catenin to drive the differentiation of peripheral tissue DC that share developmental pathways with splenic $CD8\alpha^+$ DC.

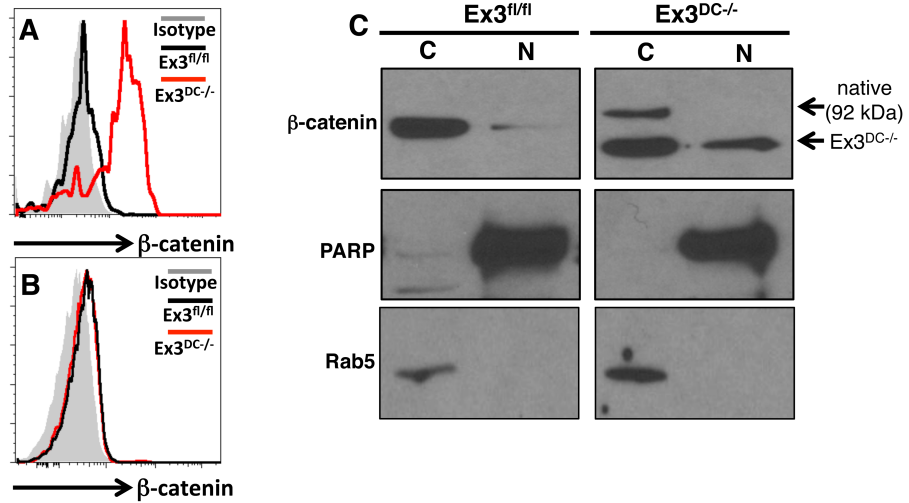


Figure 4.1. $Ex3^{DC-/-}$ mice display DC-specific accumulation and nuclear translocation of β -catenin. (A) Single cell suspensions were prepared for flow cytometry from naïve $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ spleens, and intracellular β -catenin expression was assessed after gating on $CD11c^{+}$ cells. (B) Intracellular β -catenin levels in splenic $CD4^{+}$ T cells isolated from $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ mice. (C) Bone marrow-derived DC from $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ mice were subjected to cytoplasmic (C) and nuclear (N) fractionation, and resulting lysates were probed for β -catenin protein expression. Antibodies against PARP and Rab5 were used for nuclear and cytoplasmic loading controls, respectively.

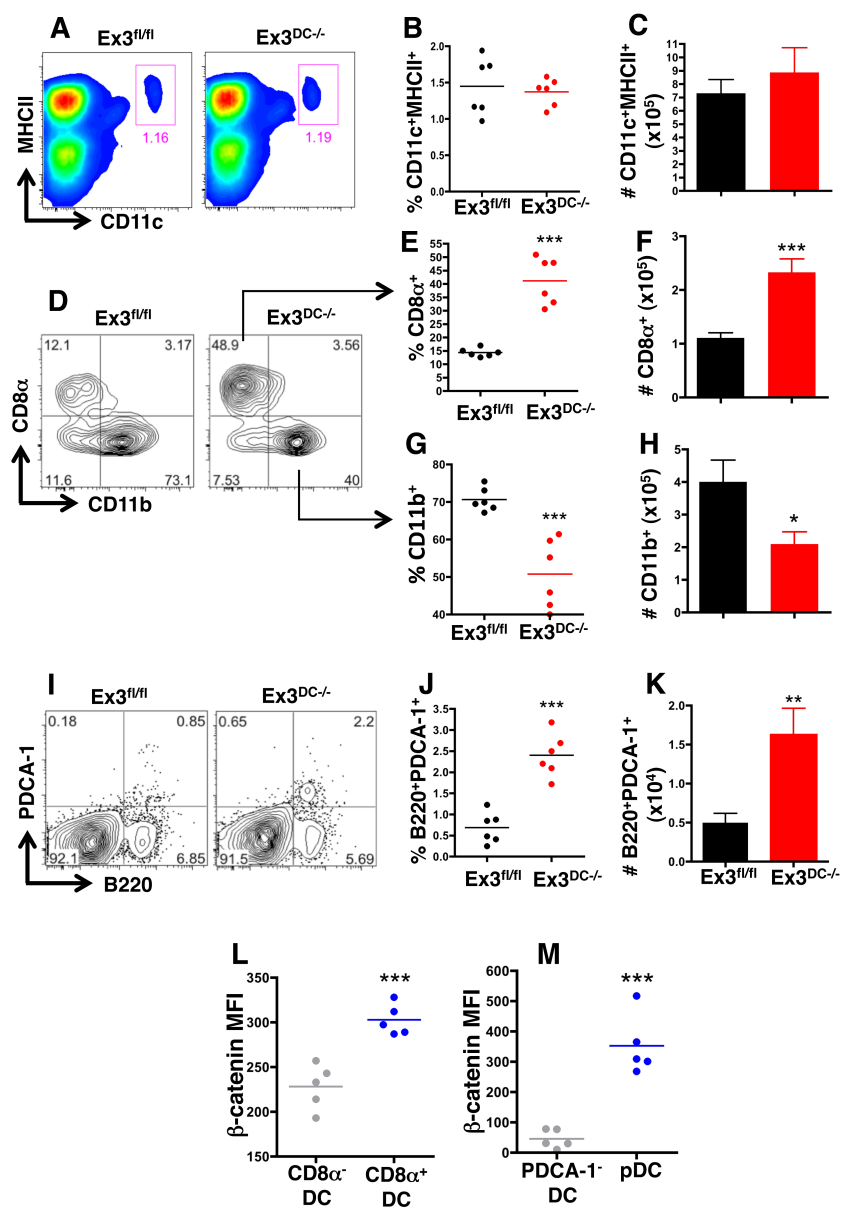


Figure 4.2. β -catenin stabilization expands splenic $CD8\alpha^+$ and plasmacytoid DC populations. (A-K) Single suspensions were prepared from naïve $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ splenocytes and stained for CD11c, MHCII, $CD8\alpha$, CD11b, PDCA-1, and B220 for DC surface marker expression analysis by flow cytometry. (A-C) Percentage and total number of $CD11c^+MHCII^+$ cells in $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ spleens. Percentage and total number of (D-F) $CD8\alpha^+$ DC and (D,G-H) $CD11b^+$ DC in naïve $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ spleens. (I-K) Percentage and total number of $B220^+PDCA-1^+$ plasmacytoid DC in naïve $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ spleens. (L and M) Splenocytes isolated from wild-type mice were surface stained for CD11c and $CD8\alpha$ (L) or PDCA-1 and B220 (M) and were intracellularly stained for β -catenin. Median fluorescent intensity (MFI) of β -catenin expression among different DC subsets is shown. Dots in relevant graphs represent results from single mice. Bar graphs display means and standard errors of individual mice. In this figure, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

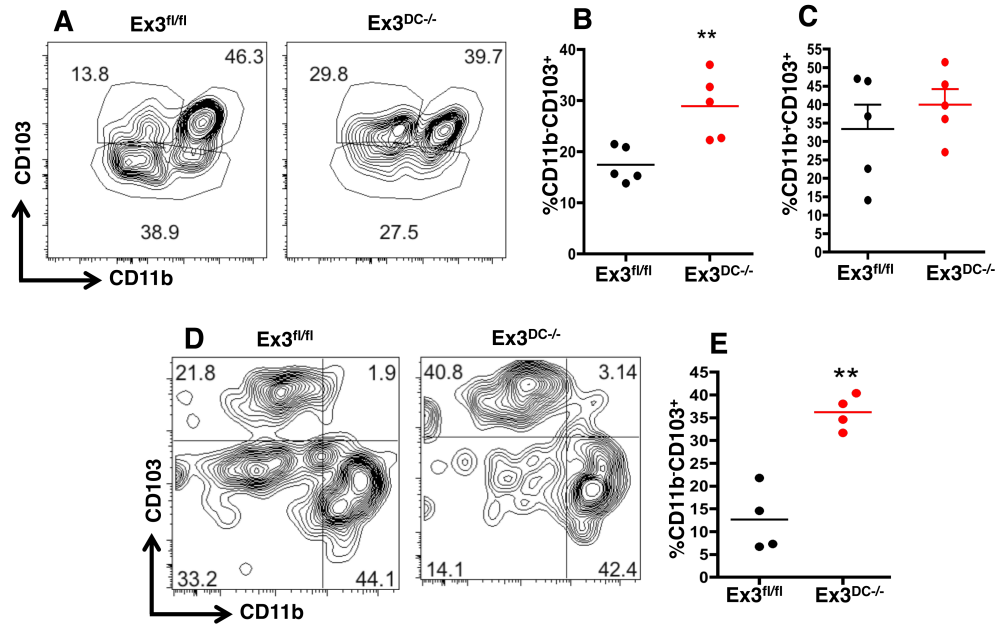


Figure 4.3 β -catenin drives the expansion of peripheral CD103⁺ DC. (A-C) Single cell suspensions were prepared from naïve Ex3^{fl/fl} and Ex3^{DC-/-} intestinal tissue following collagenase digestion and stained for CD11c, CD103, and CD11b by flow cytometry. (A) Plots from representative mice and percentages of CD103⁺CD11b⁻ (B) and CD103⁺CD11b⁺ (C) intestinal DC for multiple mice are shown. (D and E) Single cell suspensions were prepared from naïve Ex3^{fl/fl} and Ex3^{DC-/-} lung tissue and stained for CD11c, CD103, and CD11b for flow cytometric analysis of DC surface marker expression. (D) Plots from representative mice and (E) percentage of CD103⁺CD11b⁻ lung DC for multiple mice are shown. Each dot represents an individual mouse. In this figure, ** p<0.01.

Effect of β -catenin stabilization on maintenance of steady state DC

β -catenin regulates proliferation, apoptosis, and cell survival in many cell types, including hematopoietic stem cells and regulatory T cells, but its effects on DC are not known (22, 23). To address whether β -catenin affected proliferation of DC, naïve $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ mice were injected intraperitoneally with BrdU, and BrdU labeling of splenic DC was measured after 4 hr. Consistent with a role for β -catenin in promoting proliferation, S phase occupancy, as measured by BrdU uptake, was significantly increased in $CD8\alpha^+$ and $CD8\alpha^-$ DC from $Ex3^{DC-/-}$ mice compared to $Ex3^{fl/fl}$ littermate controls (Figure 4.4A-C). To measure survival, splenocytes were assayed for apoptosis and cell death by simultaneously labeling with a viability dye and Annexin V. $CD8\alpha^+$ DC from $Ex3^{DC-/-}$ mice displayed decreased Annexin V staining (Annexin V⁺, Live/Dead⁻) as well as decreased overall cell death (Annexin V⁺, Live/Dead⁺) compared to $Ex3^{fl/fl}$ littermate controls, indicating a potential increase in cell survival due to reduced apoptosis (Figure 4.4D-E). Interestingly, $Ex3^{DC-/-}$ $CD8\alpha^-$ DC of both genotypes equally labeled with Annexin and viability dye, suggesting β -catenin signaling specifically prevented apoptosis in $CD8\alpha^+$ DC (Figure 4.4D and F). Therefore, β -catenin stabilization results in increased cell proliferation and decreased apoptosis of splenic $CD8\alpha^+$ DC.

β -catenin signaling controls *Irf8* expression

Genetic knockout and gene mapping studies have identified several transcription factors involved in $CD8\alpha^+$ DC differentiation, including *Id2*, *Nfil3*, *Batf3*, and *Irf8* (5,

6, 24-27). Therefore, we determined the effect of β -catenin stabilization on expression of these transcription factors in CD11c⁺ splenocytes from Ex3^{fl/fl} and Ex3^{DC-/-} mice. While there was no significant difference in *Id2* expression, *Nfil3* and *Batf3* transcripts were slightly increased albeit in a statistically non-significant manner (Figure 4.5A). However, there was a striking increase in *Irf8* expression in the CD11c compartment upon β -catenin stabilization (Figure 4.5A). We then assessed IRF8 protein expression in CD8 α ⁻ and CD8 α ⁺ splenic DC in Ex3^{fl/fl} and Ex3^{DC-/-} mice by flow cytometry. Levels of IRF8 were relatively low in CD8 α ⁻ DC from both mouse strains. However, there was an increase in IRF8 median fluorescence intensity (MFI) in CD8 α ⁺ DC in both mouse strains (Figure 4.5B and C). Furthermore, IRF8 MFI and percent positive populations were increased when comparing CD8 α ⁺ DC from Ex3^{DC-/-} relative to Ex3^{fl/fl} strains (Figure 4.5C and D). To confirm that the effect of β -catenin stabilization was specific for IRF8, we examined expression of IRF4 and found it to be unchanged in Ex3^{DC-/-} relative to Ex3^{fl/fl} CD8 α ⁺ splenic DC (Figure 4.5E). Consistent with this finding, levels of intestinal CD103⁺CD11b⁺ DC and splenic CD4⁺ DC, which are both known to depend on IRF4 for development (7, 28), were unchanged between Ex3^{fl/fl} and Ex3^{DC-/-} mice (Figure 4.3C and 4.5F-G).

To show a direct effect of β -catenin signaling on IRF8 expression, WT BMDC were cultured with the Wnt/ β -catenin inhibitor ICG-001, which competes with β -catenin for binding to its co-factor Creb-binding protein (CBP) (29). Confirming inhibition of the Wnt/ β -catenin pathway, transcript levels of the known β -catenin target gene *Axin2*

were significantly diminished following 5 hr of culture with ICG-001 (30) (Figure 4.5H). Furthermore, *Irf8* transcripts were significantly reduced following ICG treatment. This downregulation was also observed at the protein level, as IRF8 expression in BMDC was markedly decreased compared to DMSO-treated control cells by flow cytometry, but as expected, there was no effect on total β -catenin expression (Figure 4.5I). IRF4 levels were unchanged by ICG-001 treatment, confirming the specificity of β -catenin signaling for IRF8 (data not shown). These data establish a functional link between β -catenin and IRF8 expression. The finding that β -catenin controls IRF8 expression is in line with our observation that CD8 α^+ DC, pDC, and CD103 $^+$ tissue DC are increased in Ex3^{DC-/-} mice, insofar as IRF8 is implicated in DC differentiation towards both CD8 α^+ and pDC subsets (31), and is consistent with the finding that IRF8, but not Id2, Nfil3, or Batf3, is essential for CD8 α^+ DC development (32).

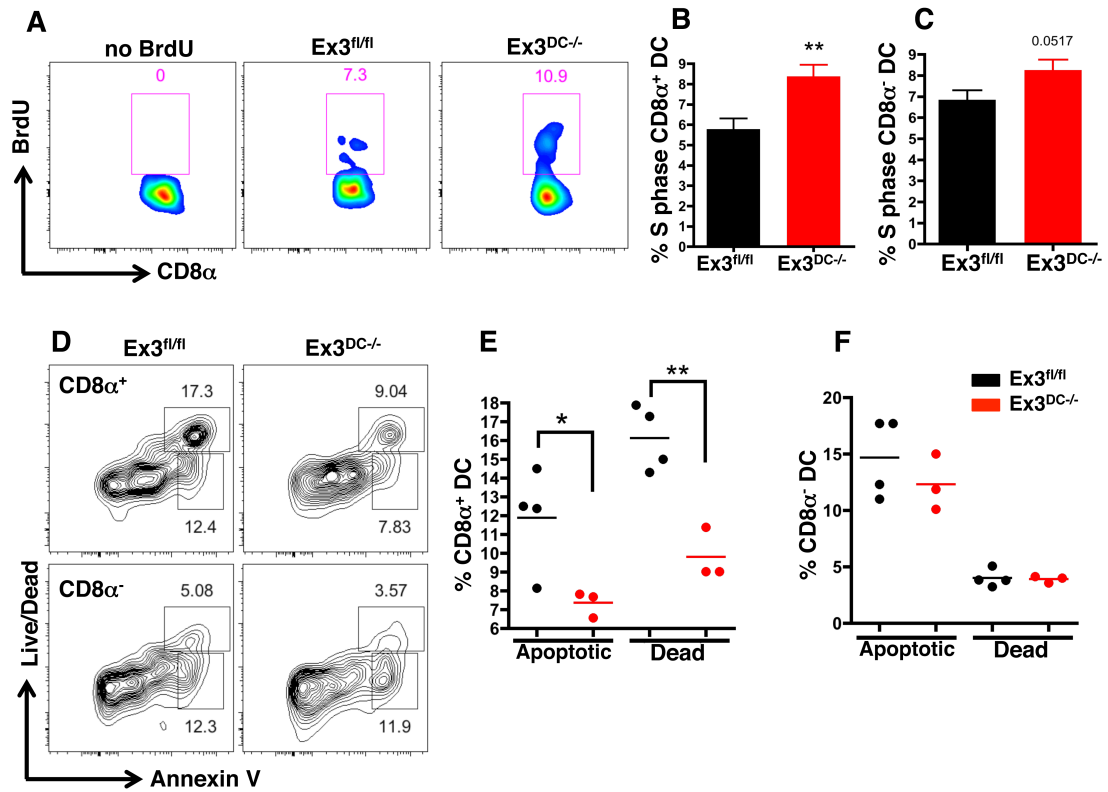


Figure 4.4. Effect of β -catenin stabilization on maintenance of steady state DC.

(A-C) Ex3^{fl/fl} and Ex3^{DC-/-} mice were injected i.p. with 2 mg bromodeoxyuridine (BrdU), and splenocytes were isolated 4 hr later. (A) Representative FACS plots of CD8 α ⁺ DC from Ex3^{fl/fl} and Ex3^{DC-/-} mice following BrdU injection. Percentage of Ex3^{fl/fl} and Ex3^{DC-/-} CD8 α ⁺ (B) and CD8 α ⁻ (C) DC occupying S phase (Ex3^{fl/fl}, n=11; Ex3^{DC-/-}, n=8). (D-F) Splenocytes were prepared from naïve Ex3^{fl/fl} and Ex3^{DC-/-} mice and were co-labeled with viability dye and Annexin V. (D) Representative FACS plots of CD8 α ⁺ and CD8 α ⁻ DC from Ex3^{fl/fl} and Ex3^{DC-/-} mice. Percentage of Ex3^{fl/fl} and Ex3^{DC-/-} CD8 α ⁺ (E) and CD8 α ⁻ (F) DC that are apoptotic (Annexin V⁺; Live/Dead⁻) or non-viable (Annexin V⁺; Live/Dead⁺) (Ex3^{fl/fl}, n=4; Ex3^{DC-/-}, n=3). In this figure, means and standard errors of individual mice are shown, where * p<0.05; ** p<0.01.

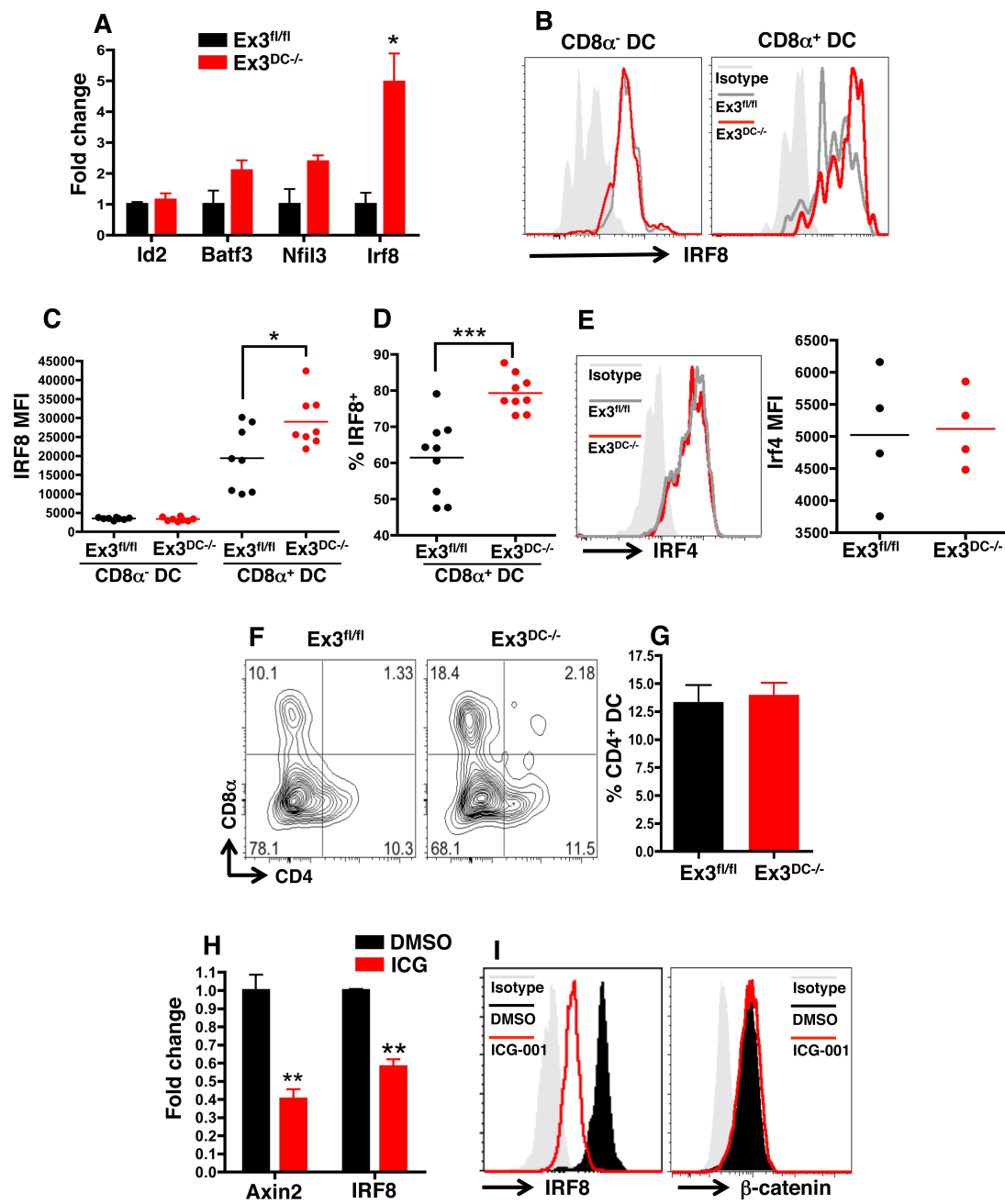


Figure 4.5. β -catenin signaling controls *Irf8* expression. (A) RNA was isolated from CD11c⁺ splenocytes from naïve Ex3^{fl/fl} and Ex3^{DC-/-} mice (n=3 per strain), and mRNA levels of *Nfil3*, *Batf3*, *Id2*, and *Irf8* were determined by semi-quantitative real-time PCR amplification and normalized to GAPDH expression. (B) IRF8 expression by Ex3^{fl/fl} and Ex3^{DC-/-} CD8 α ⁻ and CD8 α ⁺ splenic DC as determined by flow cytometry. The median fluorescent intensity (MFI) of IRF8 within Ex3^{fl/fl} and Ex3^{DC-/-} CD8 α ⁻ and CD8 α ⁺ DC (C) and the percentage of CD8 α ⁺ DC expressing IRF8 (D) are shown. (E) Ex3^{fl/fl} and Ex3^{DC-/-} CD11c⁺ splenocytes were evaluated for intracellular IRF4 expression and MFI. (F-G) CD8 α ⁻CD4⁺ splenic DC were quantified from Ex3^{fl/fl} and Ex3^{DC-/-} mice. Representative FACS plots (F) and the means and standard errors of CD4⁺ DC levels for multiple mice (G) are shown (Ex3^{fl/fl}, n=16; Ex3^{DC-/-}, n=14). (H-I) Bone marrow-derived DC were cultured with vehicle alone (DMSO) or 5 μ M of the Wnt/ β -catenin inhibitor ICG-001 for 5 hr. (H) RNA was isolated from cultured BMDC, and *Irf8* and *Axin2* transcript levels were assessed by quantitative PCR. Fold change is relative to DMSO control. (I) Intracellular expression of IRF8 and β -catenin were then assessed by flow cytometry. One representative of 4 independent experiments is shown. In this figure, * p<0.05; ** p<0.01; *** p<0.001.

Regulatory T cells are expanded upon DC-specific β -catenin stabilization

CD8 α^+ DC and DC-specific β -catenin expression have both been implicated in controlling Treg development and tolerance (1, 13, 33). To examine if β -catenin-stabilized DC have the capacity to promote tolerogenic T cell accumulation, splenic Treg levels were quantified in Ex3^{fl/fl} and Ex3^{DC-/-} mice. There was an approximately two-fold increase in the peripheral Treg population in spleens of Ex3^{DC-/-} mice compared to littermate controls both with regards to percentage and total Treg numbers (Figure 4.6A and B; data not shown). Furthermore, this phenotype was also apparent in natural Treg, as thymic Treg levels were similarly expanded in Ex3^{DC-/-} mice (Figure 4.6A and B). These data indicate that β -catenin stabilization in DC promotes their effectiveness at inducing both natural and inducible Treg populations.

β -catenin stabilization enhances IL-12 production by CD8 α^+ DC

TLR signaling most often leads to the secretion of cytokines via the induction of NF κ B transcription factors (34). However, CD8 α^+ DC were recently shown to require IRF8 rather than NF κ B for the induction of IL-12 in response to the *Toxoplasma gondii* actin polymerizing protein profilin, occurring in dependence upon TLR11/12 signaling (35). Additionally, while LPS signaling is mediated through TLR4-dependent NF κ B activation, LPS has been shown to upregulate *Irf8* expression and promote its binding to the IL-12 promoter (36, 37). Therefore, we wanted to determine if overexpression of IRF8 in CD8 α^+ DC from Ex3^{DC-/-} mice would impact their functional activity, in particular as related to IL-12 production.

To examine this, whole splenocytes from Ex3^{fl/fl} and Ex3^{DC-/-} mice were cultured in the presence of media, LPS, or soluble tachyzoite antigen (STAg), an antigenic preparation of *T. gondii* that contains profilin. Indeed, upon LPS or STAg stimulation, supernatants from Ex3^{DC-/-} splenocytes contained significantly increased levels of IL-12p40 compared to Ex3^{fl/fl} controls (Figure 4.7A). To confirm that the source of IL-12 was DC, CD11c⁺ cells were magnetically purified from Ex3^{fl/fl} and Ex3^{DC-/-} splenocytes and cultured with LPS and STAg. As expected, increased IL-12 secretion was again observed in Ex3^{DC-/-} cells, establishing that DC were the source of enhanced IL-12 production (Figure 4.7B). These results clearly show that DC from Ex3^{DC-/-} produce more IL-12 than cells from wild-type littermates. However, they leave open to question whether this is a result of an increase in the proportion of CD8 α ⁺ DC, or whether Ex3^{DC-/-} CD8 α ⁺ DC produce increased IL-12 on a cell-to-cell basis relative to corresponding cells from Ex3^{fl/fl} mice. Therefore, CD8 α ⁺ and CD8 α ⁻ DC were magnetically purified from Ex3^{fl/fl} and Ex3^{DC-/-} spleens and stimulated in vitro with STAg. While IL-12 production was restricted to the CD8 α ⁺ subset, the Ex3^{DC-/-} CD8 α ⁺ DC secreted enhanced IL-12 levels compared to Ex3^{fl/fl} CD8 α ⁺ DC (Figure 4.7C). Next, WT splenocytes were treated with ICG-001 and then stimulated with STAg or LPS overnight. Inhibitor-treated cells secreted lower levels of IL-12p40 compared to DMSO-treated cells without negatively affecting viability (Figure 4.7D and data not shown). Furthermore, the elevated IL-12 secretion displayed by Ex3^{DC-/-} splenocytes over Ex3^{fl/fl} splenocytes could be inhibited by treatment with ICG-001, further indicating that ICG-001 treatment suppresses β -catenin-dependent responses

(Figure 4.7E). Thus, β -catenin stabilization promotes both differentiation and IL-12-secreting capacity of $\text{CD8}\alpha^+$ DC by promoting increased *Irf8* expression.

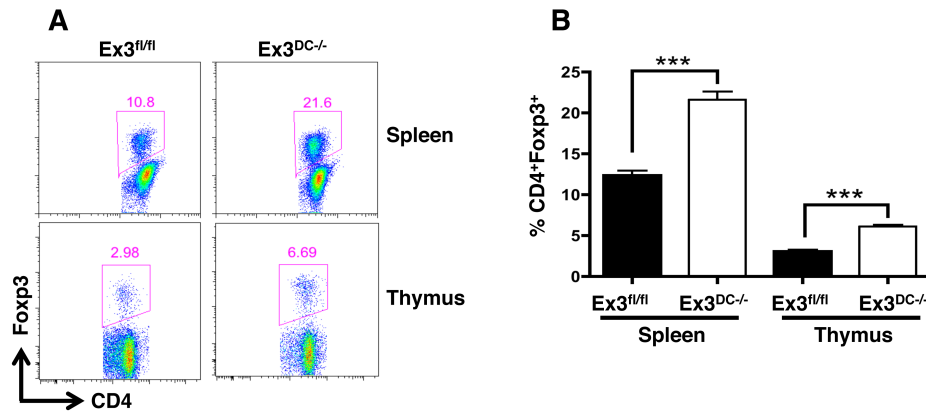


Figure 4.6 Regulatory T cells are expanded upon DC-specific β -catenin stabilization. (A) Single cell suspensions were prepared from naïve Ex3^{fl/fl} and Ex3^{DC-/-} splenocytes and thymocytes, and Fcpx3 expression was assessed amongst CD4⁺ T cells by flow cytometry to determine proportion of inducible and natural regulatory T cells (Treg), respectively. (B) The means and standard errors of natural and inducible Treg levels in splenocytes and thymocytes from individual mice. (Spleen: Ex3^{fl/fl}: n=5; Ex3^{DC-/-}: n=5; Thymus: Ex3^{fl/fl}: n=3; Ex3^{DC-/-}: n=3). In this figure, *** p<0.001.

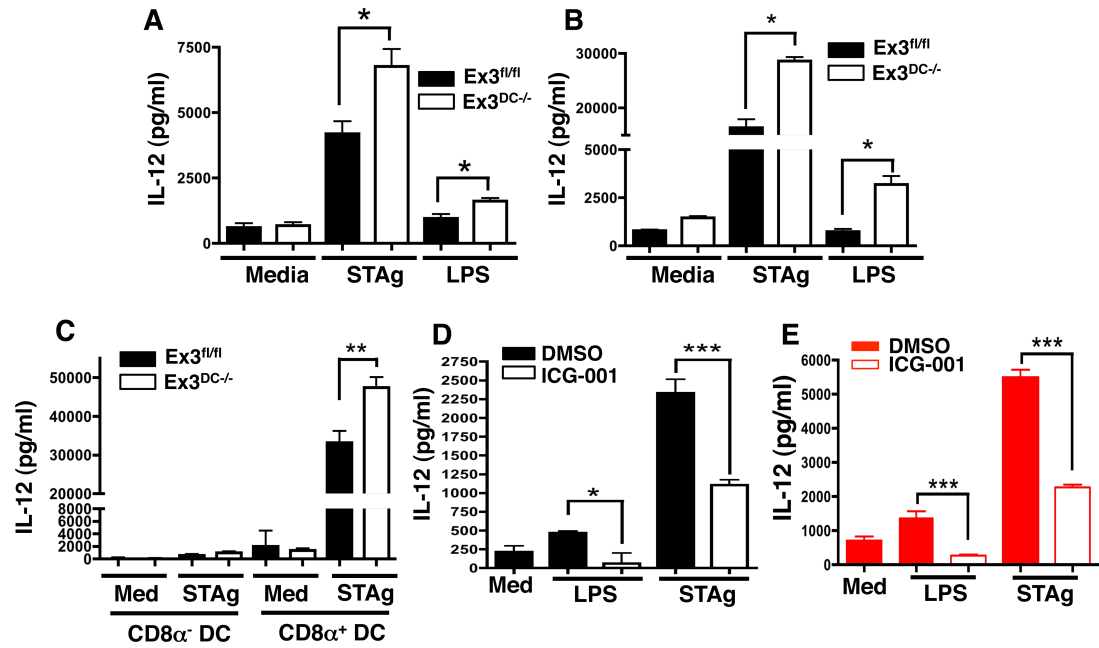


Figure 4.7. β -catenin stabilization enhances IL-12 production by CD8 α^+ DC. (A) Naïve Ex3^{fl/fl} and Ex3^{DC-/-} splenocytes were stimulated in vitro with LPS (100 ng/ml), STAg (50 mg/ml), or media, and IL-12 was measured in the supernatants by ELISA. Ex3^{fl/fl}, n=3; Ex3^{DC-/-}, n=5. (B) CD11c⁺ DC were purified from Ex3^{fl/fl} and Ex3^{DC-/-} splenocytes and stimulated in vitro with LPS, STAg, or media. IL-12 levels were measured from supernatants by ELISA. (C) CD8 α^+ DC were purified from naïve Ex3^{fl/fl} and Ex3^{DC-/-} splenocytes, and CD8 α^+ and CD8 α^- DC were stimulated in vitro with media or STAg for 48 hr. IL-12 was measured from the supernatants by ELISA. (D and E) IL-12 secretion was measured from supernatants of Ex3^{fl/fl} (D) and Ex3^{DC-/-} (E) splenocytes cultured with ICG-001 or DMSO for 5 hr and then stimulated overnight with STAg or LPS. One representative mouse of 5 with similar results is shown. In this figure, * p<0.05; ** p<0.01.

Constitutive DC β -catenin signaling renders mice susceptible to *Toxoplasma gondii* infection

To evaluate the impact of DC β -catenin stabilization on host immunity, we utilized intraperitoneal infection with *T. gondii*. CD8 α^+ DC are required for parasite control in this model as a source of protective IL-12 (18), yet overexpression of proinflammatory molecules is also lethal (19). The selective increase in CD8 α^+ DC in Ex3^{DC-/-} mice was maintained after infection, although there was an approximately 5-fold increase in absolute numbers of each population (Figure 4.8A and B, compare to Figure 4.2F). The Ex3^{DC-/-} mice began to succumb within 9 days of low dose *T. gondii* intraperitoneal infection, while the Ex3^{fl/fl} littermate controls fully survived acute infection (Figure 4.9A). There was no difference in survival of Ex3^{fl/fl} and Ex3^{DC-/-} mice following oral infection with a lethal dose of *T. gondii* (50 cysts) (Figure 4.9B); however, Ex3^{DC-/-} mice were more susceptible to oral infection with a low dose of parasite (20 cysts) (Figure 4.9C). Parasite levels in the spleen (Figure 4.9D and E) and peritoneal cavity (Figure 4.9E) were equivalent between the genotypes following low dose i.p. infection, arguing that susceptibility of Ex3^{DC-/-} mice was not due to defective control of *Toxoplasma*.

Since naïve Ex3^{DC-/-} splenic DC displayed increased IL-12 production during in vitro STAg stimulation, we examined the status of splenic DC IL-12 production during in vivo infection. CD11c⁺ DC were magnetically purified from Day-6 infected Ex3^{fl/fl} and Ex3^{DC-/-} mice and cultured without additional stimulation. Indeed, splenic DC from infected Ex3^{DC-/-} mice secreted dramatically more IL-12 compared to Ex3^{fl/fl}

controls as measured by both p40 (Figure 4.9F) and p70 (Figure 4.9G) subunits and, at nearly 50 ng/ml, the levels detected in Ex3^{DC-/-} mice were approximately 5-fold over WT controls (Figure 4.9G). Consistent with observations in naïve DC, DC from infected Ex3^{DC-/-} mice also displayed increased IRF8 expression compared to Ex3^{fl/fl} littermate controls (Figure 4.8C). To examine Th1 skewing in these animals at the peak of *T. gondii* infection, Ex3^{fl/fl} and Ex3^{DC-/-} splenocytes from Day 9-infected mice were cultured in vitro, and the supernatants were assayed for both IL-12 and IFN- γ . Accompanying increased IL-12 secretion by Ex3^{DC-/-} splenocytes, IFN- γ levels were enhanced, suggesting elevated T and possibly NK cell activation in Ex3^{DC-/-} mice in response to *T. gondii* infection (Figure 4.9H). Furthermore, serum collected from mice 9 days post-infection revealed significantly elevated levels of IFN- γ , IL-12, and TNF- α in Ex3^{DC-/-} mice compared to Ex3^{fl/fl} controls (Figure 4.9I), suggesting systemic hyperproduction of potentially lethal cytokines upon DC β -catenin stabilization.

The observation that infection of Ex3^{DC-/-} mice led to IL-12 overproduction raised the question of why elevated Treg cells that we observed (Figure 4.6) did not exert an immunomodulatory effect. However, we found that while Treg were elevated in non-infected Ex3^{DC-/-} mice, over the course of infection the population collapsed to that found in WT mice (Figure 4.8D). The collapse in Treg populations is consistent with previous studies (38, 39), and the data here show that this phenomenon occurs even in the presence of DC β -catenin stabilization that promotes CD8 α^+ DC expansion.

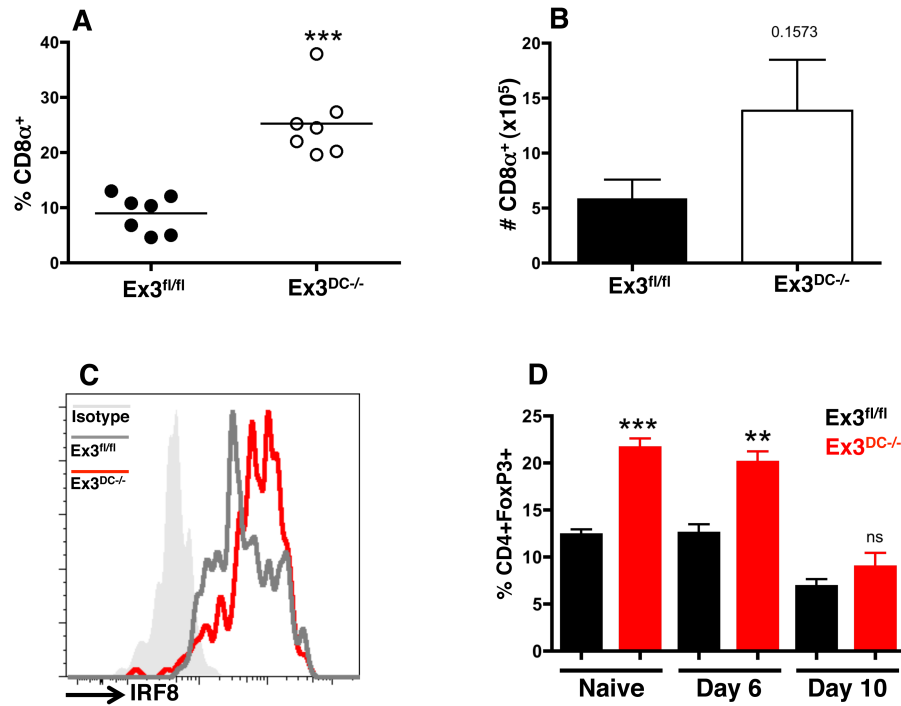


Figure 4.8. Influence of infection on levels of splenic CD8 α^+ DC and Treg populations in Ex3^{DC-/-} mice. (A-C) Ex3^{fl/fl} and Ex3^{DC-/-} mice were i.p. infected with 25 ME49 *T. gondii* cysts, and splenocytes were harvested 6 (A and B) or 9 (C) days post-infection. The percentage (A) and total number (B) of CD8 α^+ DC was determined in Ex3^{fl/fl} and Ex3^{DC-/-} spleens during infection. The means and standard errors are shown, and each dot represents an individual mouse. (C) IRF8 expression was compared between Ex3^{fl/fl} and Ex3^{DC-/-} splenic CD8 α^+ DC during infection. (D) Ex3^{fl/fl} and Ex3^{DC-/-} mice were infected (25 ME49 *T. gondii* cysts), and levels of splenic regulatory T cells (Treg), as defined by CD4 and FoxP3 expression, were evaluated by flow cytometry over a time course of infection.

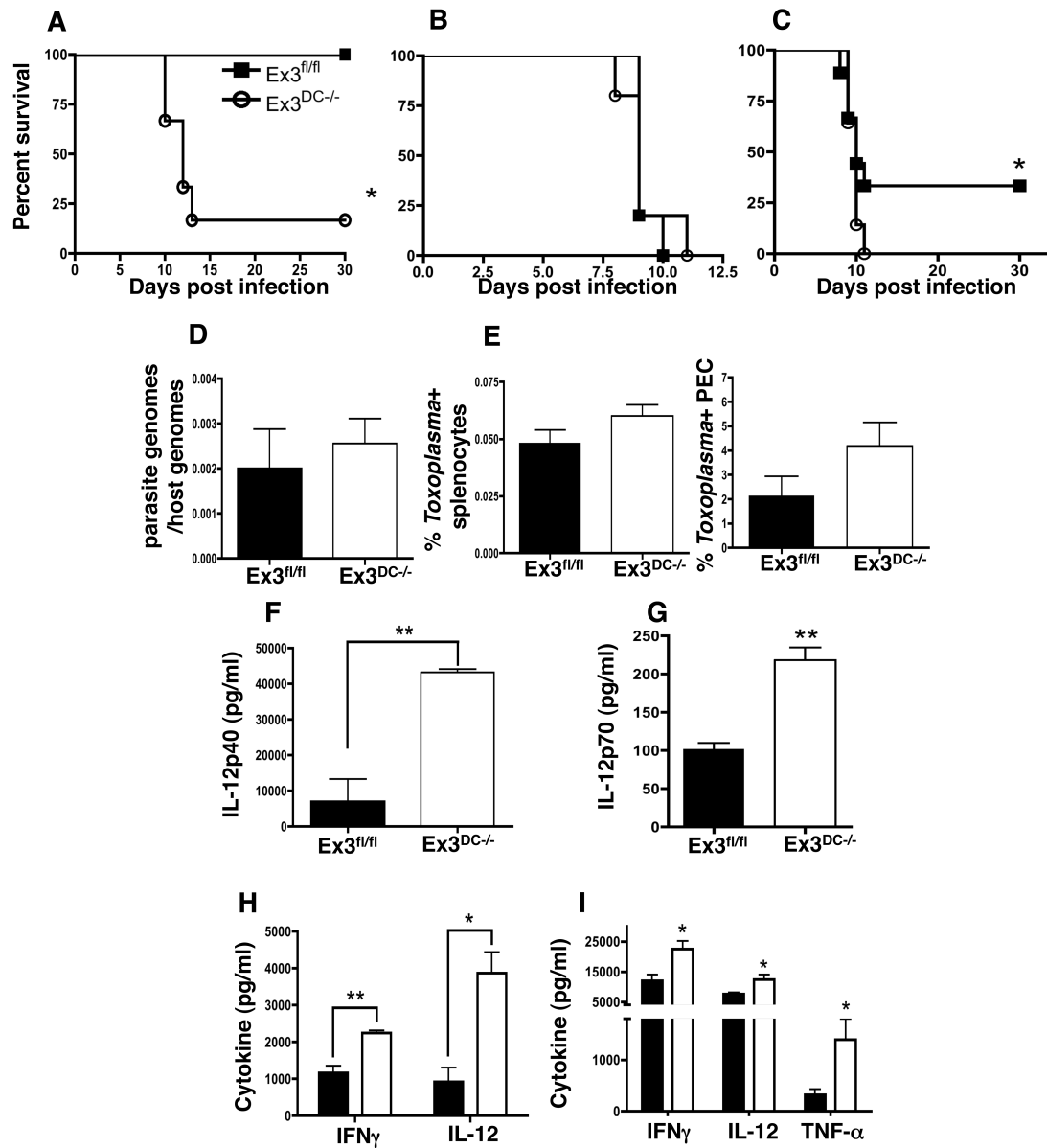


Figure 4.9. Constitutive DC β -catenin signaling renders mice susceptible to *Toxoplasma* infection. (A) $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ mice were i.p. injected with the *Toxoplasma* Type II strain ME49 (25 cysts), and survival was monitored. (B and C) $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ mice were orally infected with 50 (B) or 20 (C) ME49 *T. gondii* cysts, and survival was monitored. (D) DNA was isolated from $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ spleens 9 days post-infection, and parasite (B1 gene) and host DNA (ASL gene) were amplified by quantitative real-time PCR to determine parasite burden. Parasite load is displayed as a ratio of parasite genomes to host genomes ($\text{Ex3}^{\text{fl/fl}}$, n=4; $\text{Ex3}^{\text{DC-/-}}$, n=3). (E) Parasite burden was measured from the spleens and peritoneal cavities of Day-6 post-infection mice by flow cytometry using an antibody against *T. gondii* SAG-1 (p30) (Spleen, $\text{Ex3}^{\text{fl/fl}}$, n=3; $\text{Ex3}^{\text{DC-/-}}$, n=4. PEC, $\text{Ex3}^{\text{fl/fl}}$, n=9; $\text{Ex3}^{\text{DC-/-}}$, n=14). (F) CD11c^+ DC were purified from Day-6 post-infection splenocytes, and DC were cultured overnight without additional stimulation. IL-12p40 was measured from the supernatants by ELISA. (G) Splenocytes from Day-6 post-infection mice were cultured without additional stimulation for 72 hr, and supernatants were assayed for IL-12p70 ($\text{Ex3}^{\text{fl/fl}}$, n=3; $\text{Ex3}^{\text{DC-/-}}$, n=5). (H) Splenocytes from Day-10 post-infection mice were cultured without additional stimulation for 72 hr, and supernatants were assayed for IL-12p40 and IFN- γ by ELISA (n=3 per strain). (I) Serum was collected from Day-9 post-infection mice and assayed for IL-12p40, IFN- γ , and TNF- α by ELISA (n=3 per strain). The means and standard errors of individual mice are shown, where * p<0.05; ** p<0.01.

CD4⁺ T cells and NK cells, but not CD8⁺ T cells, overproduce IFN- γ following infection in mice with constitutive DC β -catenin signaling

To determine the source of elevated Th1 cytokines in Ex3^{DC-/-} mice during *T. gondii* infection, IFN- γ production by splenic CD4⁺ T cells, CD8⁺ T cells, and natural killer (NK) cells was assayed 8 days post-infection. Both CD4⁺ T cells and NK cell populations in Ex3^{DC-/-} mice displayed a marked increase in IFN- γ responses compared to Ex3^{fl/fl} littermate controls (Figure 4.10A and C). Interestingly, given previous studies implicating CD8 α ⁺ DC in cross-presentation (6, 18, 40), IFN- γ from CD8⁺ T cells was not significantly changed between the two genotypes (Figure 4.10B).

We also asked whether T cells activated in the Ex3^{DC-/-} environment showed signs of increased activation. However, we detected no difference in the expression of markers of proliferation (Ki67) on CD4⁺ and CD8⁺ T cells during infection (Figure 4.11A) or in expression of the costimulatory molecules CD80 and CD86 on DC (data not shown). In support of this, OVA-pulsed splenic DC from Ex3^{fl/fl} and Ex3^{DC-/-} mice stimulated equivalent OT-II CD4⁺ T cell proliferation in vitro (Figure 4.11B).

We next determined whether the increase in the CD4⁺ T cell IFN- γ -positive population could be explained by increased activation and expansion of *Toxoplasma*-specific T cells. We used MHC I tetramers that bind to CD8⁺ T lymphocytes specific for the endogenous *T. gondii* epitope Tgd057 and MHC II tetramers that bind to CD4⁺ T cells specific for the *Toxoplasma* epitope CD4Ag28m (41, 42). As shown in Figures 4.10D and E, the frequency of parasite-specific CD4⁺ and CD8⁺ T cells was equivalent in

Ex3^{DC-/-} and Ex3^{fl/fl} mice. Furthermore, while tetramer-positive cells of both genotypes demonstrated enhanced IFN- γ secretion over tetramer-negative cells, antigen-specific CD4⁺ T cells from Ex3^{DC-/-} mice secreted markedly higher levels of IFN- γ relative to Ex3^{fl/fl} counterparts (Figure 4.10F and G).

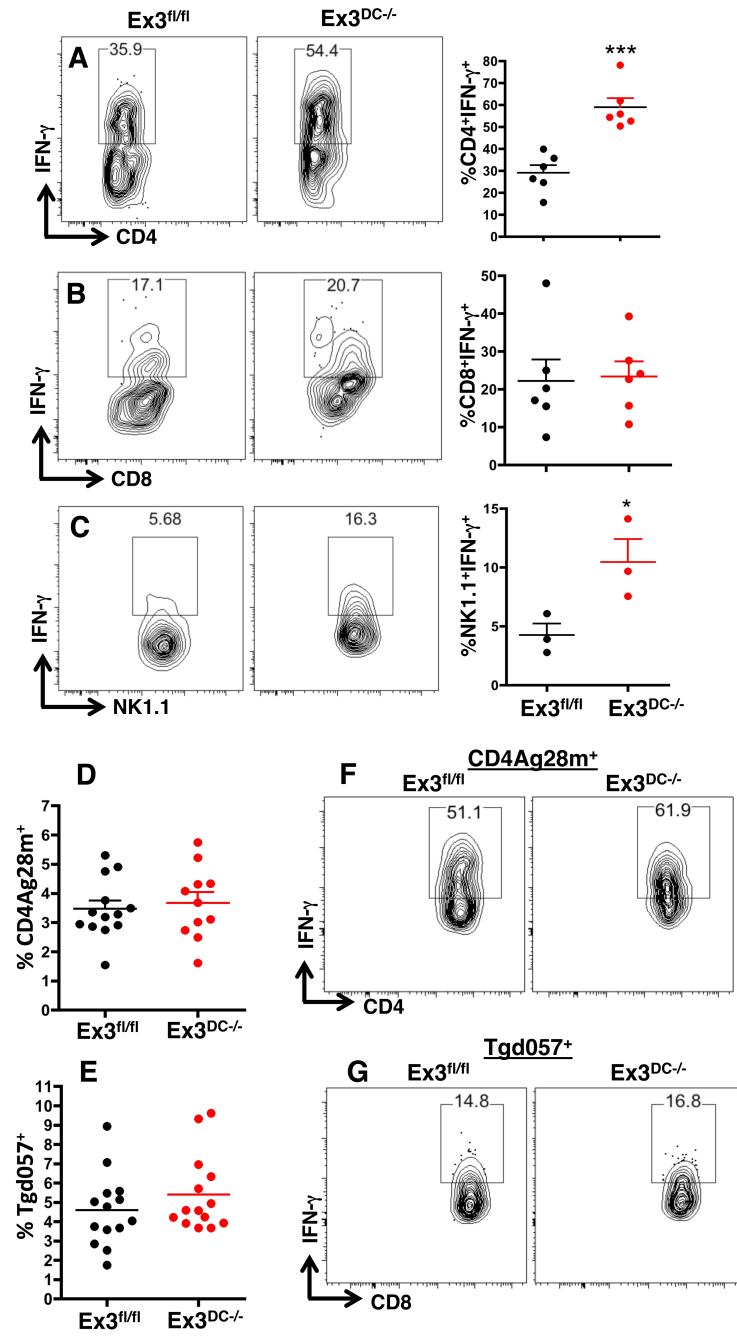


Figure 4.10. CD4⁺ T cells and NK cells, but not CD8⁺ T cells, overproduce IFN- γ following infection in mice with constitutive DC β -catenin signaling. (A-C) Single cell suspensions were prepared from Ex3^{fl/fl} and Ex3^{DC-/-} spleens 9 days post-infection, and cells were cultured in the presence of PMA, ionomycin, and Brefeldin-A for 4 hr. Cells were surface stained for CD4 (A), CD8 (B), or NK1.1 (C) and intracellularly stained for IFN- γ . Shown are representative contour plots of individual mice and the mean \pm standard error of IFN- γ levels from multiple mice. (D-G) Splenocyte single cell suspensions from Day 9-infected Ex3^{fl/fl} and Ex3^{DC-/-} mice were surface stained with tetramers specific for the MHC class II *T. gondii* epitope CD4Ag28m (D) and the MHC class I epitope Tgd057 (E) recognized by parasite-specific CD4⁺ and CD8⁺ T cells, respectively, to assess antigen-specific T cell priming. Tetramer-positive CD4⁺ (F) and CD8⁺ (G) T cells were then assessed for IFN- γ levels following 4 hr of culture with PMA, ionomycin, and Brefeldin-A (Ex3^{fl/fl}, n=3; Ex3^{DC-/-}, n=3). Means and standard errors of individual mice are shown, and each dot represents a single mouse. In this figure, * p<0.05; *** p<0.001.

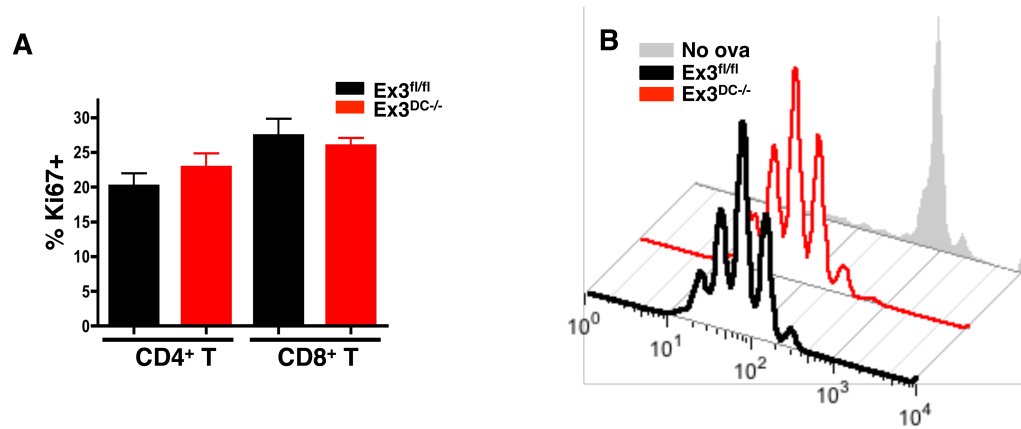


Figure 4.11. Constitutive DC β -catenin signaling does not influence antigen-specific T cell proliferative responses. (A) Ex3^{fl/fl} and Ex3^{DC-/-} mice were infected i.p. with 25 ME49 *T. gondii* cysts, and spleens were harvested after 6 days. Splenocytes were surface stained for CD4 or CD8 and intracellularly for Ki67 to assess T cell proliferation by flow cytometry. (B) Splenic DC were magnetically sorted from naïve Ex3^{fl/fl} and Ex3^{DC-/-} mice and pulsed with OVA peptide for 4 hr. OVA-pulsed DC (10^5) were then co-cultured with CFSE-labeled OT-II CD4⁺ T cells for 72 hr (2.5: 1 T cell: DC). CFSE dilution was examined by flow cytometry to determine T cell proliferation.

Discussion

Dendritic cell differentiation is a complex process involving an array of transcription factors and growth factor cytokines whose details are continuing to be elucidated. In this study we identify an unexpected role for β -catenin in controlling expansion of $CD8\alpha^+$ DC, pDC, and developmentally related non-lymphoid $CD103^+$ DC. This pattern of transcriptional control precisely matches that of IRF8 (2, 21, 25, 28, 31). In support of a functional connection between β -catenin and IRF8, overexpression of β -catenin increased steady state IRF8 levels in $CD8\alpha^+$ DC. In addition, IL-12 production by $CD8\alpha^+$ DC, a known function of this cell subset, was prevented by β -catenin inhibition concomitant with IRF8 down-modulation, and $CD8\alpha^+$ DC overexpressing β -catenin produced enhanced IL-12 in response to microbial stimulation. During infection with *T. gondii*, a prototypic Th1-inducing pathogen, mice overexpressing DC-specific β -catenin developed exacerbated proinflammatory cytokine responses culminating in early death. Consistent with established roles for β -catenin in other cell types, such as hematopoietic stem cells and regulatory T cells (22, 23), β -catenin stabilization also resulted in increased proliferation and resistance to apoptosis by $CD8\alpha^+$ splenic DC, suggesting a potential two-pronged mechanism of β -catenin-dependent $CD8\alpha^+$ DC expansion.

Our data are notable because other recent studies have implicated β -catenin signaling in promoting tolerogenic DC phenotypes. For example, in contrast to activation by microbial stimulation, cluster disruption of bone marrow-derived DC activates β -

catenin, endowing the cells with the ability to promote regulatory T cells that protect against experimental autoimmune encephalitis (13, 43). In the intestine, β -catenin expression by CD11c⁺ cells was shown to be required for regulatory T cell induction and production of anti-inflammatory factors, including retinoic acid-metabolizing enzymes, TGF- β , and IL-10 (1). In this case, absence of β -catenin in DC increased sensitivity to dextran sodium sulfate-mediated colitis. Along parallel lines, DC-specific β -catenin expression is also involved in tumor-mediated inhibition of CD8⁺ T cell cross priming (14). Our data uncover an important new facet of β -catenin signaling because they reveal a role in promoting the differentiation and survival of IRF8-dependent CD8 α ⁺ DC with enhanced proinflammatory activity. Consistent with our findings, a recent study also identified a functional link between Wnt/ β -catenin and IRF8 in leukemia progression, whereby Tcf/Lef1 transcription factors can directly bind the *Irf8* promoter at one of three consensus bindings sites, providing an independent line of evidence for the importance of this previously unappreciated signaling axis (44).

Although our findings reveal an overall proinflammatory effect of DC-specific β -catenin activation, our findings are also concordant with earlier studies insofar as DC-specific β -catenin stabilization increased steady state levels of Foxp3⁺ Treg. Consistent with the expansion of CD8 α ⁺ DC in our model, these cells have been implicated in the induction of Treg (33). We hypothesized that the induction of Treg by DC β -catenin stabilization would result in protection from the *T. gondii*-mediated

intestinal inflammation that follows high dose oral infection (45). Surprisingly, $\text{Ex3}^{\text{DC-/-}}$ mice were equally susceptible to high dose infection with *Toxoplasma* and were more susceptible to both oral and intraperitoneal low dose infections that are normally non-lethal. In fact, the expanded steady-state Treg population is unlikely to impact control of *T. gondii* because infection induces rapid collapse of this T cell subset, as was observed by us in the present study and previously by others (38, 39). Therefore, by its unique property of minimizing Treg numbers, *T. gondii* infection exposed a novel inflammatory role for β -catenin that was likely concealed by experimental models in which Treg, play a functional role, such as DSS colitis and EAE (1, 13). It will be interesting to investigate whether $\text{Ex3}^{\text{DC-/-}}$ mice are protected from pathologies that can be prevented by Treg in future studies.

Several lines of evidence indicate a role for $\text{CD8}\alpha^+$ DC in antigen cross presentation and activation of CD8^+ T lymphocytes (6, 40, 46). *Toxoplasma* is well known for its ability to elicit potent CD8^+ T cell responses, and cross presentation has previously been found to play a role in MHC class I presentation to CD8^+ T cells during infection with this intracellular protozoan (47-49). Therefore, it was initially surprising that there was no indication of abnormally strong CD8^+ T cell responses in infected $\text{Ex3}^{\text{DC-/-}}$ mice that overexpress $\text{CD8}\alpha^+$ DC. However, the recent discovery that the parasite directly injects a subset of secretory proteins into the host cell cytoplasm (50-52), as well as evidence that the majority of T cell activation is stimulated by actively infected DC (53), argues that conventional presentation rather than cross presentation may be the dominant mechanism for CD8^+ T cell priming.

In contrast to normal CD8⁺ T cell responses in mice with DC-specific β -catenin activation, there was a clear increase in CD4⁺ T cell, and to a lesser extent NK cell, IFN- γ production after infection of the mutant mice. This is of interest because CD4⁺ and CD11b⁺ DC, which are believed to be the most adept at activating CD4⁺ T cells (54, 55), were unchanged or even reduced in Ex3^{DC-/-} mice. While it seems most likely that increased CD4⁺ T and NK cell IFN- γ results from increased splenic CD8 α ⁺ DC activity, we cannot at this point exclude a role for pDC that are also increased. Indeed, pDC have previously been shown to present antigen and produce IL-12 during *Toxoplasma* infection (56). We also found that pDC are preferentially targeted for infection in the spleen (57). Finally, we cannot exclude the possibility that CD11c⁺ inflammatory monocytes might mediate increased CD4⁺ T cell activation upon β -catenin stabilization. Regardless of these uncertainties, our data demonstrate that β -catenin signaling underlies IRF8-dependent differentiation and function of DC subsets.

Our findings uncover for the first time a role for stabilized β -catenin signaling in promoting DC subset differentiation and activity. Based upon previous findings, it has been suggested that exploiting strategies that activate β -catenin signaling in DC might be useful in the control of inflammatory and autoimmune diseases (1, 13). Our study throws a cautionary light on this approach, showing that constitutive β -catenin signaling alters the steady state DC differentiation program by acting through IRF8 to skew these cells towards a proinflammatory phenotype.

Materials and methods

Ethics statement

All experiments in this study were performed strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at Cornell University (permit number 1995-0057). All efforts were made to minimize animal suffering during the course of these studies.

Mice and infections

Female Swiss Webster mice (6-8 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME), and female C57BL/6 were purchased from Taconic Farms (Germantown, NY). C57BL/6-Tg(Tcr α Tcr β)425Cbn/J (OT-II) mice were obtained from the Jackson Laboratory and maintained as a breeding colony at Cornell University College of Veterinary Medicine. The β -catenin Ex3^{fl/fl} mice (43) were kindly provided by M. M. Taketo (Kyoto University) were maintained as breeding colonies crossed to CD11c-cre expressing mice at the Transgenic Mouse Core Facility at the Cornell University College of Veterinary Medicine. Cre⁺ offspring (Ex3^{DC/-} mice) were identified by PCR amplification of the *Cre* gene from genomic DNA isolated from tail snips. Mouse infections were initiated in 8-12 week old mice by intraperitoneal or oral inoculation of 25 cysts of the type II *T. gondii* ME49 strain. Cysts were isolated from chronically infected Swiss Webster mice by homogenization of whole brain in sterile PBS.

Preparation and purification of leukocytes

Splenocyte single cell suspensions were prepared by crushing spleens between sterile glass slides and filtering the resulting suspension through 40 μ M filters. For lung leukocytes, lung tissue was minced with sterile razor blades and incubated with collagenase type IV (Sigma) in a 37°C water bath for 30 min with frequent agitation. The resulting digest was passed through a 40 μ M filter to create a single cell suspension. Bone marrow-derived DC were cultured as described previously (58). Briefly, femurs of C57BL/6 mice were flushed with PBS and cultured for 9 days in media containing 10% fetal calf serum (Hyclone), 100 U/ml penicillin (Life Technologies), 0.1 mg/ml streptomycin (Life Technologies), 50 μ M 2-mercaptoethanol (Sigma), and 20 ng/ml GM-CSF (Peprotech). A single round of positive selection using CD11c⁺ magnetic bead sorting was performed for purification of total splenic DC from single cell suspensions (Stem Cell Technologies), while two-step magnetic bead sorting, with an initial negative selection to enrich for DC followed by CD8 α ⁺ positive selection (Miltenyi Biotec), was performed to isolate CD8 α ⁺ splenic DC.

Western blotting

To validate nuclear translocation of β -catenin in Ex3^{DC-/-} mice, BMDC were subjected to nuclear and cytoplasmic fractionation following the manufacturer's guidelines (Active Motif). Resulting nuclear and cytoplasmic proteins were diluted in reducing SDS sample buffer and separated by 10% SDS-PAGE. Separated proteins were transferred onto nitrocellulose and blocked for 1 hr at room temperature in Tris-

buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk (TBST). Following 3 washes in TBST, blots were incubated overnight in primary antibody diluted in TBST containing 5% BSA. Blots were subsequently washed in TBST and incubated for 1 hr with anti-rabbit IgG conjugated to horseradish peroxidase-conjugated (HRP) diluted in TBST containing 5% nonfat dry milk. Following 5 washes in TBST, blots were developed and imaged using a chemiluminescent detection system (Thermo Scientific). Anti- β -catenin and anti-PARP were purchased from Cell Signaling, and anti-Rab5a was purchased from Santa Cruz Biotechnology.

Flow cytometry

Single cell suspensions were washed in PBS prior to resuspension in Zombie Aqua viability dye (BioLegend) for 15 min at room temperature to exclude dead cells. Primary antibodies (anti-CD11c FITC or APC, anti-CD8 α Pacific Blue or APC-Cy7, anti-CD4 PerCP or FITC, anti-CD205 PerCP, anti-PDCA-1 APC or PE, anti-NK1.1 APC, anti-CD11b APC-Cy7, anti-CD103 PE, anti-B220 PE) resuspended in ice-cold FACS buffer (1% bovine serum albumin/0.01% NaN₃ in PBS) were added directly to the cells for 30 min. Tetramer staining for Tgd057⁺CD8⁺ T cells (provided by George Yap, New Jersey School of Medicine and Dentistry) and CD4Ag28m⁺CD4⁺ T cells (provided by Marion Pepper, University of Washington) was performed by labeling at room temperature for 1 hour. For intracellular staining, cells were fixed using the FoxP3/transcription factor staining kit fixative (eBioscience) and subsequently incubated with primary antibodies resuspended in the FoxP3/transcription factor permeabilization buffer (eBioscience). Antibodies used for intracellular cytokine

staining include anti-IRF8 PerCP, anti-FoxP3 APC, anti-Ki67 APC, and anti-IRF4 e450 (eBioscience); anti- β -catenin Alexa-647 (Cell Signaling); and anti-*Toxoplasma gondii* (Argene) For IFN- γ staining, cells were incubated for 4 hrs with Brefeldin-A (eBioscience; 10 μ g/ml), PMA (Sigma; 10 ng/ml), and ionomycin (Sigma; 1 μ g/ml), then fixed with the FoxP3/transcription factor staining kit fixative (eBioscience) and subsequently incubated with anti-IFN- γ (PE-Cy7; BioLegend). All samples were run on an LSRII flow cytometer (BD), and the data were analyzed using FlowJo software (FlowJo, Ashland, OR).

Splenic DC BrdU and Annexin labeling

Mice were injected with 2 mg BrdU (Sigma), and spleens were harvested 4 hours later. Single cell suspensions were subjected to BrdU labeling using a FITC BrdU labeling kit following the manufacturer's instructions (BD). For Annexin labeling, cells were co-stained for Annexin V (eBioscience) and Zombie Aqua viability dye (BioLegend) following the manufacturer's instructions for the FITC Annexin V kit (eBioscience).

ICG-001 inhibition

BMDC (1×10^6) were plated and cultured overnight. The following day, the cells were cultured with 5 μ M ICG-001 (Selleck Chemicals) or DMSO control for 5 hr at 37°C. Cells were surface stained for CD11c, fixed, intracellularly stained for IRF8, IRF4, and β -catenin, and analyzed by flow cytometry. Alternatively, RNA was harvested from cultured cells, and transcriptional analysis was performed for *Irf8* and *Axin2* expression by quantitative PCR.

Measurement of mRNA by quantitative PCR

RNA was isolated from CD11c⁺ splenocytes magnetically sorted from naïve Ex3^{DC-/-} and Ex3^{fl/fl} mice by resuspension in Trizol reagent (Life Technologies). RNA was converted to cDNA (Quanta Biosciences, Gaithersburg, MD) and assayed for gene expression by SYBR green technology (Quanta Biosciences). Primers were designed to span exons by Integrated DNA Technologies. The following primer sequences were used:

<i>Irf8</i>	Forward,	TGCCACTGGTGACCGGATAT;	Reverse,
		GACCATCTGGGAGAAAGCTGAA;	
		<i>Nfil3</i>	Forward,
		GAACTCTGCCTTAGCTGAGGT;	Reverse,
		ATTCCCGTTTTCTCCGACACG;	
		<i>Id2</i>	Forward,
		ATGAAAGCCTTCAGTCCGGTG;	Reverse,
		AGCAGACTCATCGGGTCGT;	
		<i>Batf3</i>	Forward,
		CAGACCCAGAAGGCTGACAAG;	Reverse,
		CTGCGCAGCACAGAGTTCTC;	
<i>Axin2</i>	Forward,	TAGGTTCCGGCTATGTCTTTG;	Reverse,
		TGTTTCTTACTCCCCATGCG.	

GAPDH was used as a housekeeping gene. Gene expression was normalized to Ex3^{fl/fl} samples or DMSO controls.

Cytokine measurement

IFN- γ , IL-12p70, and TNF- α secretion were assayed by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (eBioscience) following culture with media or soluble tachyzoite antigen (50 μ g/ml) prepared as previously described (48). IL-12p40 secretion was measured using an in-house ELISA (59).

Parasite burden measurement

Levels of *T. gondii* DNA were measured as described previously (60). Briefly, spleens were homogenized, and DNA was extracted using a tissue extraction kit (Omega Biotech). The *T. gondii* B1 gene and the host argininosuccinate lyase (ASL) gene were amplified by quantitative real-time PCR, and resulting ct values were compared to standard curves developed from 10-fold serial dilutions of parasite DNA and splenocyte DNA, respectively. The parasite burden is displayed as the ratio of *T. gondii* DNA to host DNA.

CFSE proliferation assay

Splenic DC (2×10^5) purified from $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ mice by CD11c^+ positive selection were pulsed with ovalbumin (OVA) for 4 hrs. OVA-specific CD4^+ T cells were harvested from spleens of naïve OT-II/ $\text{Rag1}^{-/-}$ mice and labeled with CFSE following the manufacturer's instructions (Invitrogen). CFSE-labeled OT-II T cells were then co-cultured with OVA-pulsed DC (2.5:1 T cell : DC) for 3 days. CFSE dilution was measured by flow cytometry to assess T cell proliferation.

Statistical analyses

Differences between groups were analyzed by Student's *t* test. A Kaplan-Meier curve (Logrank test) was used to calculate differences in survival between $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ mice. P values were considered statistically significant at <0.05 and were designated *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

References

1. Manicassamy, S., B. Reizis, R. Ravindran, H. Nakaya, R. M. Salazar-Gonzalez, Y. C. Wang, and B. Pulendran. 2010. Activation of β -Catenin in Dendritic Cells Regulates Immunity Versus Tolerance in the Intestine. *Science* 329: 849–853.
2. Belz, G. T., and S. L. Nutt. 2012. Transcriptional programming of the dendritic cell network. *Nat Rev Immunol* 12: 101–113.
3. Manicassamy, S., and B. Pulendran. 2011. Dendritic cell control of tolerogenic responses. *Immunol. Rev.* 241: 206–227.
4. Satpathy, A. T., W. KC, J. C. Albring, B. T. Edelson, N. M. Kretzer, D. Bhattacharya, T. L. Murphy, and K. M. Murphy. 2012. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *Journal of Experimental Medicine* 209: 1135–1152.
5. Jackson, J. T., Y. Hu, R. Liu, F. Masson, A. D'Amico, S. Carotta, A. Xin, M. J. Camilleri, A. M. Mount, A. Kallies, L. Wu, G. K. Smyth, S. L. Nutt, and G. T. Belz. 2011. Id2 expression delineates differential checkpoints in the genetic program of CD8 α ⁺ and CD103⁺ dendritic cell lineages. *EMBO J.* 30: 2690–2704.
6. Hildner, K., B. T. Edelson, W. E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, R. D. Schreiber, T. L. Murphy, and K. M. Murphy. 2008. Batf3 deficiency reveals a critical role for CD8 α ⁺ dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097–1100.
7. Persson, E. K., H. Uronen-Hansson, M. Semmrich, A. Rivollier, K. Hägerbrand, J. Marsal, S. Gudjonsson, U. Håkansson, B. Reizis, K. Kotarsky, and W. W. Agace. 2013. IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* 38: 958–969.
8. Meredith, M. M., K. Liu, G. Darrasse-Jeze, A. O. Kamphorst, H. A. Schreiber, P. Guernonprez, J. Idoyaga, C. Cheong, K.-H. Yao, R. E. Niec, and M. C. Nussenzweig. 2012. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *Journal of Experimental Medicine* 209: 1153–1165.
9. Reya, T., A. W. Duncan, L. Ailles, J. Domen, D. C. Scherer, K. Willert, L. Hintz, R. Nusse, and I. L. Weissman. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 423: 409–414.
10. Clevers, H., and R. Nusse. 2012. Wnt/ β -catenin signaling and disease. *Cell* 149: 1192–1205.
11. Klaus, A., and W. Birchmeier. 2008. Wnt signalling and its impact on

development and cancer. *Nat. Rev. Cancer* 8: 387–398.

12. Staal, F. J. T., T. C. Luis, and M. M. Tiemessen. 2008. WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol* 8: 581–593.

13. Jiang, A., O. Bloom, S. Ono, W. Cui, J. Unternaehrer, S. Jiang, J. A. Whitney, J. Connolly, J. Banchereau, and I. Mellman. 2007. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27: 610–624.

14. Liang, X., C. Fu, W. Cui, J. L. Ober-Blöbaum, S. P. Zahner, P. A. Shrikant, B. E. Clausen, R. A. Flavell, I. Mellman, and A. Jiang. 2014. β -catenin mediates tumor-induced immunosuppression by inhibiting cross-priming of CD8⁺ T cells. *J. Leukoc. Biol.* 95: 179–190.

15. Harada, N., Y. Tamai, T. Ishikawa, B. Sauer, K. Takaku, M. Oshima, and M. M. Taketo. 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* 18: 5931–5942.

16. Denkers, E. Y., and R. T. Gazzinelli. 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin. Microbiol. Rev.* 11: 569–588.

17. Dupont, C. D., D. A. Christian, and C. A. Hunter. 2012. Immune response and immunopathology during toxoplasmosis. *Semin Immunopathol* 34: 793–813.

18. Mashayekhi, M., M. M. Sandau, I. R. Dunay, E. M. Frickel, A. Khan, R. S. Goldszmid, A. Sher, H. L. Ploegh, T. L. Murphy, L. D. Sibley, and K. M. Murphy. 2011. CD8 α (+) dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites. *Immunity* 35: 249–259.

19. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kühn, W. Müller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4⁺ T cells and accompanied by overproduction of IL-12, IFN- γ and TNF- α . *J. Immunol.* 157: 798–805.

20. Hall, A. O., D. P. Beiting, C. Tato, B. John, G. Oldenhove, C. G. Lombana, G. H. Pritchard, J. S. Silver, N. Bouladoux, J. S. Stumhofer, T. H. Harris, J. Grainger, E. D. T. Wojno, S. Wagage, D. S. Roos, P. Scott, L. A. Turka, S. Cherry, S. L. Reiner, D. Cua, Y. Belkaid, M. M. Elloso, and C. A. Hunter. 2012. The cytokines interleukin 27 and interferon- γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity* 37: 511–523.

21. Edelson, B. T., W. KC, R. Juang, M. Kohyama, L. A. Benoit, P. A. Klekotka, C. Moon, J. C. Albring, W. Ise, D. G. Michael, D. Bhattacharya, T. S. Stappenbeck, M. J.

- Holtzman, S.-S. J. Sung, T. L. Murphy, K. Hildner, and K. M. Murphy. 2010. Peripheral CD103⁺ dendritic cells form a unified subset developmentally related to CD8 α ⁺ conventional dendritic cells. *Journal of Experimental Medicine* 207: 823–836.
22. Scheller, M., J. Huelsken, F. Rosenbauer, M. M. Taketo, W. Birchmeier, D. G. Tenen, and A. Leutz. 2006. Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat. Immunol.* 7: 1037–1047.
23. Ding, Y., S. Shen, A. C. Lino, M. A. Curotto de Lafaille, and J. J. Lafaille. 2008. Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. *Nat. Med.* 14: 162–169.
24. Kashiwada, M., N.-L. L. Pham, L. L. Pewe, J. T. Harty, and P. B. Rothman. 2011. NFIL3/E4BP4 is a key transcription factor for CD8 α ⁺ dendritic cell development. *Blood* 117: 6193–6197.
25. Aliberti, J., O. Schulz, D. J. Pennington, H. Tsujimura, C. Reis e Sousa, K. Ozato, and A. Sher. 2003. Essential role for ICSBP in the in vivo development of murine CD8 α ⁺ dendritic cells. *Blood* 101: 305–310.
26. Hacker, C., R. D. Kirsch, X.-S. Ju, T. Hieronymus, T. C. Gust, C. Kuhl, T. Jorgas, S. M. Kurz, S. Rose-John, Y. Yokota, and M. Zenke. 2003. Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat. Immunol.* 4: 380–386.
27. Jaiswal, H., M. Kaushik, R. Sougrat, M. Gupta, A. Dey, R. Verma, K. Ozato, and P. Tailor. 2013. Batf3 and Id2 Have a Synergistic Effect on Irf8-Directed Classical CD8 α ⁺ Dendritic Cell Development. *The Journal of Immunology* 191: 5993–6001.
28. Tamura, T., P. Tailor, K. Yamaoka, H. J. Kong, H. Tsujimura, J. J. O'Shea, H. Singh, and K. Ozato. 2005. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J. Immunol.* 174: 2573–2581.
29. Emami, K. H., C. Nguyen, H. Ma, D. H. Kim, K. W. Jeong, M. Eguchi, R. T. Moon, J.-L. Teo, S. W. Oh, H. Y. Kim, S. H. Moon, J. R. Ha, and M. Kahn. 2004. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. *Proc. Natl. Acad. Sci. U.S.A.* 101: 12682–12687.
30. Jho, E.-H., T. Zhang, C. Domon, C.-K. Joo, J.-N. Freund, and F. Costantini. 2002. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol. Cell. Biol.* 22: 1172–1183.
31. Schiavoni, G., F. Mattei, P. Sestili, P. Borghi, M. Venditti, H. C. Morse, F. Belardelli, and L. Gabriele. 2002. ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8 α (+)

dendritic cells. *J. Exp. Med.* 196: 1415–1425.

32. Seillet, C., J. T. Jackson, K. A. Markey, H. J. M. Brady, G. R. Hill, K. P. A. Macdonald, S. L. Nutt, and G. T. Belz. 2013. CD8 α ⁺ DCs can be induced in the absence of transcription factors Id2, Nfil3, and Batf3. *Blood* 121: 1574–1583.

33. Yamazaki, S., D. Dudziak, G. F. Heidkamp, C. Fiorese, A. J. Bonito, K. Inaba, M. C. Nussenzweig, and R. M. Steinman. 2008. CD8⁺ CD205⁺ splenic dendritic cells are specialized to induce Foxp3⁺ regulatory T cells. *The Journal of Immunology* 181: 6923–6933.

34. Dunne, A., and L. A. J. O'Neill. 2003. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci. STKE* 2003: re3.

35. Raetz, M., A. Kibardin, C. R. Sturge, R. Pifer, H. Li, E. Burstein, K. Ozato, S. Larin, and F. Yarovinsky. 2013. Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to *Toxoplasma gondii* profilin. *The Journal of Immunology* 191: 4818–4827.

36. Barber, S. A., M. J. Fultz, C. A. Salkowski, and S. N. Vogel. 1995. Differential expression of interferon regulatory factor 1 (IRF-1), IRF-2, and interferon consensus sequence binding protein genes in lipopolysaccharide (LPS)-responsive and LPS-hyporesponsive macrophages. *Infection and Immunity* 63: 601–608.

37. Xu, H., J. Zhu, S. Smith, J. Foldi, B. Zhao, A. Y. Chung, H. Outtz, J. Kitajewski, C. Shi, S. Weber, P. Saftig, Y. Li, K. Ozato, C. P. Blobel, L. B. Ivashkiv, and X. Hu. 2012. Notch-RBP-J signaling regulates the transcription factor IRF8 to promote inflammatory macrophage polarization. *Nat. Immunol.* 13: 642–650.

38. Oldenhove, G., N. Bouladoux, E. A. Wohlfert, J. A. Hall, D. Chou, L. Dos Santos, S. O'Brien, R. Blank, E. Lamb, S. Natarajan, R. Kastenmayer, C. Hunter, M. E. Grigg, and Y. Belkaid. 2009. Decrease of Foxp3⁺ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* 31: 772–786.

39. Benson, A., S. Murray, P. Divakar, N. Burnaevskiy, R. Pifer, J. Forman, and F. Yarovinsky. 2012. Microbial infection-induced expansion of effector T cells overcomes the suppressive effects of regulatory T cells via an IL-2 deprivation mechanism. *The Journal of Immunology* 188: 800–810.

40. Haan, den, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192: 1685–1696.

41. Wilson, D. C., G. M. Grotenbreg, K. Liu, Y. Zhao, E.-M. Frickel, M.-J. Gubbels, H. L. Ploegh, and G. S. Yap. 2010. Differential regulation of effector- and central-

memory responses to *Toxoplasma gondii* Infection by IL-12 revealed by tracking of Tgd057-specific CD8⁺ T cells. *PLoS Pathog* 6: e1000815.

42. Grover, H. S., N. Blanchard, F. Gonzalez, S. Chan, E. A. Robey, and N. Shastri. 2012. The *Toxoplasma gondii* peptide AS15 elicits CD4 T cells that can control parasite burden. *Infection and Immunity* 80: 3279–3288.

43. Fu, C., and A. Jiang. 2009. Generation of tolerogenic dendritic cells via the E-cadherin/ β -catenin-signaling pathway. *Immunol Res* 46: 72–78.

44. Scheller, M., J. Schönheit, K. Zimmermann, U. Leser, F. Rosenbauer, and A. Leutz. 2013. Cross talk between Wnt/ β -catenin and Irf8 in leukemia progression and drug resistance. *Journal of Experimental Medicine* 210: 2239–2256.

45. Egan, C. E., S. B. Cohen, and E. Y. Denkers. 2012. Insights into inflammatory bowel disease using *Toxoplasma gondii* as an infectious trigger. *Immunol. Cell Biol.* 90: 668–675.

46. Pooley, J. L., W. R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8⁺ dendritic cells, but cross-presented to CD8 T cells by CD8⁺ dendritic cells. *J. Immunol.* 166: 5327–5330.

47. Goldszmid, R. S., I. Coppens, A. Lev, P. Caspar, I. Mellman, and A. Sher. 2009. Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *Toxoplasma gondii*-infected dendritic cells. *Journal of Experimental Medicine* 206: 399–410.

48. Denkers, E. Y., R. T. Gazzinelli, S. Hieny, P. Caspar, and A. Sher. 1993. Bone marrow macrophages process exogenous *Toxoplasma gondii* polypeptides for recognition by parasite-specific cytolytic T lymphocytes. *J. Immunol.* 150: 517–526.

49. John, B., T. H. Harris, E. D. Tait, E. H. Wilson, B. Gregg, L. G. Ng, P. Mrass, D. S. Roos, F. Dzierszinski, W. Weninger, and C. A. Hunter. 2009. Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with *Toxoplasma gondii*. *PLoS Pathog* 5: e1000505.

50. Melo, M. B., K. D. C. Jensen, and J. P. J. Saeij. 2011. *Toxoplasma gondii* effectors are master regulators of the inflammatory response. *Trends Parasitol* 27: 487–495.

51. Denkers, E. Y., D. J. Bzik, B. A. Fox, and B. A. Butcher. 2012. An inside job: hacking into Janus kinase/signal transducer and activator of transcription signaling cascades by the intracellular protozoan *Toxoplasma gondii*. *Infection and Immunity* 80: 476–482.

52. Taylor, S., A. Barragan, C. Su, B. Fux, S. J. Fentress, K. Tang, W. L. Beatty, H. E.

Hajj, M. Jerome, M. S. Behnke, M. White, J. C. Wootton, and L. D. Sibley. 2006. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science* 314: 1776–1780.

53. Dupont, C. D., D. A. Christian, E. M. Selleck, M. Pepper, M. Leney-Greene, G. Harms Pritchard, A. A. Koshy, S. Wagage, M. A. Reuter, L. D. Sibley, M. R. Betts, and C. A. Hunter. 2014. Parasite Fate and Involvement of Infected Cells in the Induction of CD4⁺ and CD8⁺ T Cell Responses to *Toxoplasma gondii*. *PLoS Pathog* 10: e1004047.

54. Mount, A. M., C. M. Smith, F. Kupresanin, K. Stoermer, W. R. Heath, and G. T. Belz. 2008. Multiple dendritic cell populations activate CD4⁺ T cells after viral stimulation. *PLoS ONE* 3: e1691.

55. Vander Lugt, B., A. A. Khan, J. A. Hackney, S. Agrawal, J. Lesch, M. Zhou, W. P. Lee, S. Park, M. Xu, J. DeVoss, C. J. Spooner, C. Chalouni, L. Delamarre, I. Mellman, and H. Singh. 2014. Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nat. Immunol.* 15: 161–167.

56. Pepper, M., F. Dzierszinski, E. Wilson, E. Tait, Q. Fang, F. Yarovinsky, T. M. Laufer, D. Roos, and C. A. Hunter. 2008. Plasmacytoid dendritic cells are activated by *Toxoplasma gondii* to present antigen and produce cytokines. *J. Immunol.* 180: 6229–6236.

57. Bierly, A. L., W. J. Shufesky, W. Sukhumavasi, A. E. Morelli, and E. Y. Denkers. 2008. Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during *Toxoplasma gondii* infection. *The Journal of Immunology* 181: 8485–8491.

58. Schneider, A. G., D. S. Abi Abdallah, B. A. Butcher, and E. Y. Denkers. 2013. *Toxoplasma gondii* triggers phosphorylation and nuclear translocation of dendritic cell STAT1 while simultaneously blocking IFN γ -induced STAT1 transcriptional activity. *PLoS ONE* 8: e60215.

59. Butcher, B. A., L. Kim, P. F. Johnson, and E. Y. Denkers. 2001. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-kappa B. *J. Immunol.* 167: 2193–2201.

60. Cohen, S. B., K. J. Maurer, C. E. Egan, S. Oghumu, A. R. Satoskar, and E. Y. Denkers. 2013. CXCR3-dependent CD4⁺ T cells are required to activate inflammatory monocytes for defense against intestinal infection. *PLoS Pathog* 9: e1003706.

CHAPTER 5

Exploring the Role of β -catenin in Splenic and Bone Marrow-Derived Dendritic Cells

Abstract

β -catenin has been reported as a negative regulator of dendritic cell (DC) function by promoting tolerogenic responses, although most of these studies have been performed in vitro using bone marrow-derived (BM) DC. Previous work in our lab has indicated a pro-inflammatory role for β -catenin in tissue resident DC in vivo using a transgenic model of DC-specific constitutive β -catenin activation. In this study, we sought to examine the role of β -catenin in BMDC as compared to splenic DC using this transgenic mouse system. We find inverse roles for β -catenin in in vitro-derived BMDC and splenic DC, whereby proinflammatory IL-12 secretion is reduced and induction of antigen-specific T cell proliferation is abolished in BMDC. Surprisingly, phosphorylation of MAPK and STAT mediators was largely unaffected by β -catenin activation in BMDC. These results contrast with findings in splenic DC, where phosphorylation of p38 and STAT3 was enhanced. However, they corroborate previous findings of a tolerogenic role for β -catenin in BMDC and further shed light on the DC subset-specific role of this signaling axis in vivo versus in vitro.

Introduction

Dendritic cells (DC) are important sentinels of the immune system that, upon recognition of foreign antigen, alert cells of innate immunity to an invading pathogen through secretion of pro-inflammatory cytokines while simultaneously presenting the antigen to T cells to initiate adaptive immunity. In order to perform these tightly regulated processes, DC must respond to foreign antigens through fine tuned signaling processes downstream of cell surface receptors, leading to increased expression of major histocompatibility complexes, costimulatory molecules, signaling mediators, and migration towards lymph nodes (1). A question of immense interest has been how DC are able to distinguish between pathogen-derived antigens and self- or commensal-derived antigens, as improper immune responses against harmless antigens can lead to autoimmune diseases, such as inflammatory bowel disease (2, 3).

One recently identified player in the DC-mediated regulation of immunity versus tolerance is the Wnt/ β -catenin signaling axis. Wnt signaling is generally recognized as a regulator of tumorigenesis, as Wnt1, a member of the Wnt gene family, was originally identified as an oncogene activated by the genomic insertion of mouse mammary tumor virus (4). The pathway centers on β -catenin, a transcription factor that is normally kept at low levels in the cytoplasm by constitutive glycogen synthase kinase (GSK)- β -mediated phosphorylation and proteasomal degradation. Upon binding of an activating Wnt ligand to specific cell surface Frizzled receptors, β -catenin is released from its degrading complex and is free to enter the nucleus to activate transcription of its target genes (5). Wnt signaling has been implicated in

controlling numerous cellular functions, including cell cycle, survival, stem cell renewal, and body axis polarization (6-10). More recently, β -catenin has been implicated in DC immune processes related to tolerance induction. One particular groundbreaking finding is that deletion of β -catenin in DC leads to an emergence of proinflammatory cytokines and a loss of regulatory T cells in the intestine, implicating this signaling axis in dampening inflammatory responses in one of the most antigen rich immune compartments in the body (11). Other studies have used in vitro methods to demonstrate a tolerizing role for β -catenin, whereby disruption of cell-to-cell cadherin complexes or stimulation of DC with Wnt ligands leads to secretion of anti-inflammatory cytokines (12, 13), and activation or knockdown of β -catenin in bone marrow progenitors using targeting vectors promotes or impairs DC maturation, respectively (14). Our group more recently identified a striking reverse role for β -catenin signaling in tissue resident DC, whereby constitutive activation of β -catenin leads to expansion of certain conventional DC subsets in dependence on IRF8 signaling (Cohen et al, Manuscript in Review; See Chapter 4).

Therefore, we sought to compare the role of β -catenin in splenic and bone marrow-derived (BM) DC using the same transgenic mouse model, in which Exon 3 of the β -catenin gene is deleted in vivo by a CD11c-driven Cre recombinase, thus constitutively activating β -catenin only in CD11c-expressing cells. Most β -catenin studies have used non-infectious stimuli, such as LPS, dextran sulfate sodium, or Wnt ligands, to investigate the effect of β -catenin on BMDC function (11, 13, 14).

Therefore, we included the infectious agent *Toxoplasma gondii* as a model organism for our study. *T. gondii* is a protozoan parasite that is most often transmitted by the ingestion of tissue cysts from undercooked meat. It is a highly prevalent pathogen that infects approximately 11% of people in the United States (15) and is a strong inducer of IL-12 production, leading to a potent type I inflammatory response (16). Furthermore, it has the profound capacity to manipulate host signaling molecules to control the outcome of the immune response (17). We therefore wondered whether infection with *T. gondii* would yield similar effects on immunity as LPS following constitutive β -catenin activation.

Using both LPS and *T. gondii* as stimuli, we find that BMDC and splenic DC respond differently to constitutive β -catenin activation. Consistent with previously published reports, β -catenin signaling in BMDC provides an inhibitory effect on their ability to appropriately respond to stimulation, as measured by cytokine secretion and upregulation of costimulatory molecules. We further show that β -catenin impairs the ability of BMDC to stimulate antigen-specific T cell responses in vitro. However, splenic DC with active β -catenin signaling display increased phosphorylation of the MAP kinase p38 and STAT3 following stimulation, consistent with our previous finding of a pleiotropic role for β -catenin in DC, involving elevated cytokine potential, increased regulatory T cell activation, and conventional DC differentiation.

Results

β -catenin activation differentially controls activation and maturation of splenic and bone marrow-derived DC

Previous studies in our lab identified a role for β -catenin in promoting inflammation by splenic dendritic cells (DC) using a Cre/flox transgenic mouse model of CD11c-specific β -catenin stabilization (Ex3^{DC-/-} mice), in particular through the expansion of CD8 α^+ DC (Cohen et al, Manuscript in Review; See Chapter 4). This phenotype was determined to be a result of IRF8 upregulation, leading to increased IL-12 secretion by DC with constitutive β -catenin activation. We next asked if β -catenin contributes to the expression of signaling molecules in the mitogen-activated protein kinase (MAPK) and signal-transducer and activator of transcription (STAT) families, which are involved in the regulation of numerous cellular functions, including cytokine production and survival (18-22). To address this, CD11c⁺ splenic DC were isolated from Ex3^{DC-/-} mice and their Ex3^{fl/fl} wild-type (WT) littermate controls by magnetic separation, stimulated ex vivo with soluble tachyzoite antigen (STAg), and immunoblotted for phosphorylated ERK, p38, and STAT3. There was a small but consistent increase in the early phosphorylation of both ERK and p38 upon β -catenin activation, but while p38 maintained its phosphorylation status in both genotypes, ERK phosphorylation strongly decreased in Ex3^{DC-/-} DC after 1 hr (Figure 5.1). The differences between Ex3^{fl/fl} and Ex3^{DC-/-} DC were particularly apparent in the unstimulated cells, suggesting that β -catenin may promote phosphorylation of MAPK even in the absence of external stimulation. Additionally, STAT3 phosphorylation

occurred earlier upon activation of β -catenin phosphorylation and was clearly detectable within 15 minutes of STAg stimulation as compared to 30 minutes for WT control cells (Figure 5.1). Together these results suggest that β -catenin expression enhances phosphorylation of MAPK and STAT family members in splenic DC following ex vivo stimulation.

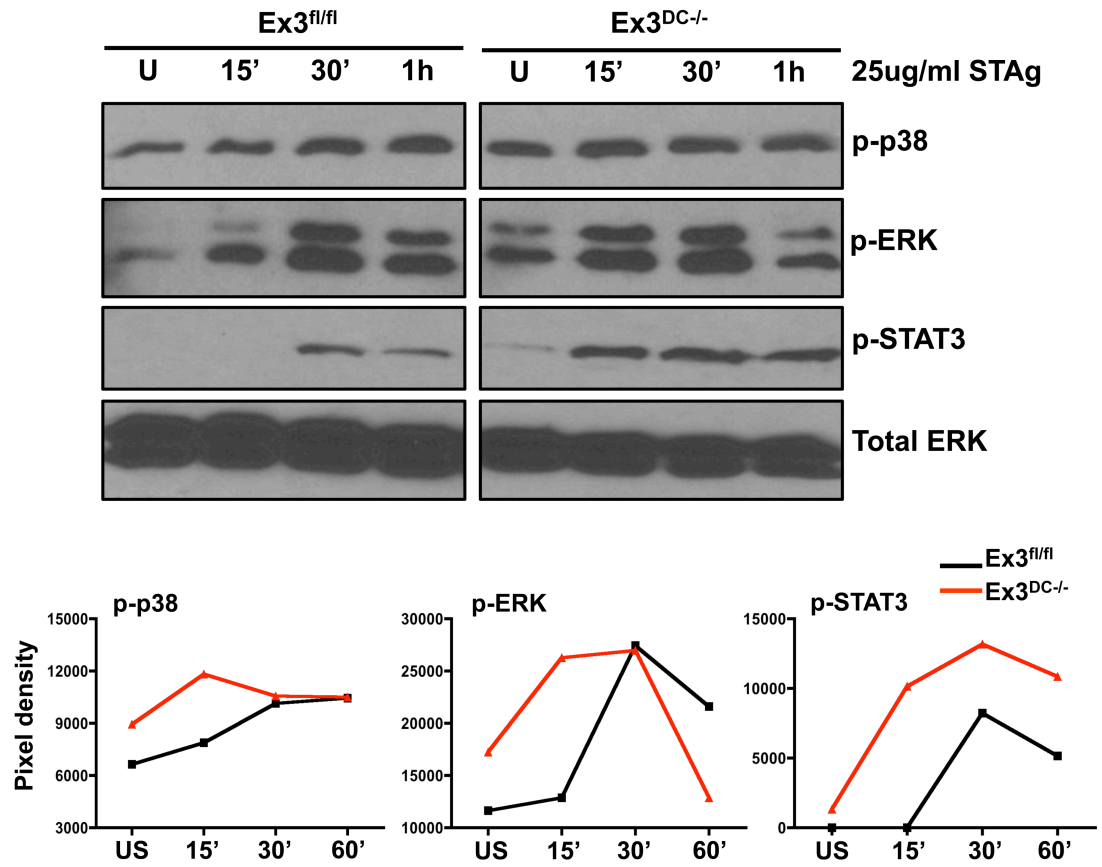


Figure 5.1. Splenic DC with stabilized β -catenin display increased ERK and STAT phosphorylation following STAg stimulation. CD11c⁺ splenic DC were magnetically purified from Ex3^{fl/fl} and Ex3^{DC-/-} mice and stimulated ex vivo with 25 μ g/ml soluble tachyzoite antigen (STAg). Lysates were harvested over 3 timepoints, and phosphorylation of p38, ERK, and STAT3 were then examined by Western blot. Total ERK was used as a loading control. Densitometry was performed to qualitatively compare pixel density of bands at each time point.

Bone marrow-derived (BM) DC represent a lineage that is distinct from the CD8 α^+ DC found in the spleen (23). Therefore, we next asked whether BMDC display a similar activation phenotype upon β -catenin stabilization. To address this, DC were generated in vitro from the bone marrow of Ex3^{fl/fl} and Ex3^{DC-/-} mice through addition of the growth factor cytokine granulocyte-macrophage colony stimulating factor (GM-CSF). Upon stimulation of Ex3^{fl/fl} and Ex3^{DC-/-} BMDC with lipopolysaccharide (LPS), there were no remarkable differences in the phosphorylation of ERK (Figure 5.2A). Furthermore, when the Type II *T. gondii* Prugniaud strain was used as a stimulus, ERK phosphorylation was again equivalent between the genotypes (Figure 5.2A). Therefore, β -catenin accumulation in BMDC does not impact ERK phosphorylation following in vitro stimulation.

The MAPK p38 is required for NF κ B-mediated IL-12 production in response to *Toxoplasma* (18), and STAT3 has been shown to regulate tolerant DC function as well as interact with the Wnt signaling pathway(22, 24). Therefore, upregulation of p38 and STAT3 phosphorylation were examined in Ex3^{fl/fl} and Ex3^{DC-/-} BMDC following stimulation with the Type I *T. gondii* strain RH. Surprisingly, BMDC from both strains displayed equivalent phosphorylation of p38 and STAT3, arguing against a role for β -catenin in controlling these signaling mediators (Figure 5.2B). We next examined the possibility that STAT1 phosphorylation was affected by β -catenin activation, as IFN- γ -dependent signaling is crucial for an optimal immune response against *Toxoplasma*. To address this, Ex3^{fl/fl} and Ex3^{DC-/-} BMDC were stimulated with

recombinant IFN- γ , and STAT1 phosphorylation was examined. Early phosphorylation was equivalent between the BMDC strains, although there was a slight decrease in sustained STAT1 phosphorylation at late timepoints in Ex3^{DC-/-} BMDC (Figure 5.2C). While the functional impact of STAT1 phosphorylation was not tested, such as induction of IFN- γ -responsive genes, this could suggest a minor impairment in STAT1-dependent signaling over time. Examination of costimulatory molecule expression following stimulation with STAg provided some additional evidence of this impaired activation; although expression levels of CD80 and CD86 were similar between Ex3^{fl/fl} and Ex3^{DC-/-} BMDC after 3 h, these levels dropped off in Ex3^{DC-/-} BMDC by the overnight timepoint (Figure 5.2D).

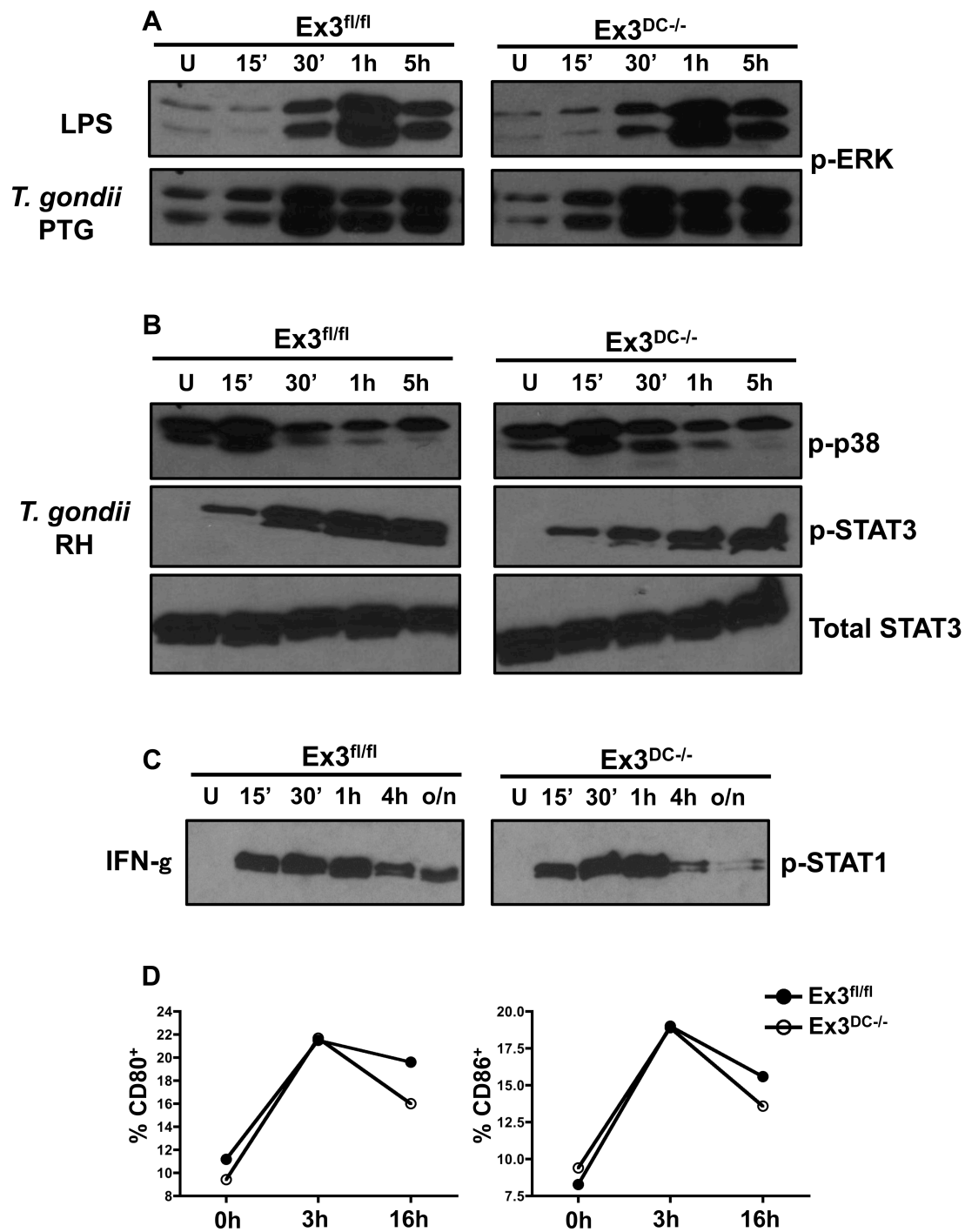


Figure 5.2. Effect of β -catenin stabilization on BMDC activation and maturation.

(A) $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ BMDC were stimulated with LPS (100 ng/ml) or the Type II *T. gondii* strain Prugniaud (Pru), and lysates were collected over multiple timepoints for analysis of ERK phosphorylation by Western blot. (B) $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ BMDC were stimulated with the Type I *T. gondii* strain RH over multiple timepoints, and lysates were examined for phosphorylation of p38 and STAT3 by Western blot. Total STAT3 was used as a loading control. (C) $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ BMDC were stimulated with recombinant IFN- γ (100 ng/ml), and lysates from multiple timepoints were assessed for tyrosine phosphorylation of STAT1 by Western blot. (D) $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ BMDC were stimulated with STAg (50 $\mu\text{g/ml}$), and cells were surface stained for costimulatory molecules CD80 and CD86 and analyzed by flow cytometry.

β -catenin activation in BMDC impairs IL-12 production

Since splenic DC with constitutively active β -catenin secrete elevated levels of IL-12 (Cohen et al, Manuscript in Review; See Chapter 4) and upregulate early p38 phosphorylation upon stimulation (Figure 5.1), we wondered whether IL-12 production by BMDC would be affected by β -catenin signaling. Therefore, BMDC generated from $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ mice were cultured with LPS or STAg overnight, and IL-12p40 levels in the cell supernatants were assessed by ELISA. Surprisingly, considering our earlier results with $CD8\alpha^+$ DC (See Chapter 4), lower levels of IL-12p40 secretion were observed in $Ex3^{DC-/-}$ BMDC cultures for both LPS and STAg stimulations, although the difference in the LPS culture did not quite reach statistical significance (Figure 5.3A). To confirm these results, BMDC cultures from $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ mice were stimulated with LPS or the Type II *T. gondii* strain PTG overnight, and Brefeldin-A was added for the last 5 hr of culture to measure intracellular levels of IL-12 by flow cytometry. A statistically significant reduction in IL-12 expression was observed in $Ex3^{DC-/-}$ BMDC compared to $Ex3^{fl/fl}$ BMDC under both LPS and Pru conditions (Figure 5.3B-C). In direct contrast to splenic DC, these results suggest that β -catenin expression in BMDC impairs secretion of IL-12 following stimulation, consistent with a tolerogenic phenotype in this lineage.

β -catenin stabilization inhibits BMDC-dependent T cell proliferation

A major role of dendritic cells is to activate T cells in an antigen-specific manner through the presentation of processed peptides. To examine the impact of β -catenin on the ability of BMDC to stimulate antigen-specific T cell responses, we utilized

transgenic T cells that are engineered to express a T cell receptor specific for ovalbumin peptide (OVA). Thus, BMDC from $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ were pulsed with OVA and then cultured with CFSE-labeled OVA-specific OT-II T cells to measure T cell proliferation. In response to BMDC generated from $\text{Ex3}^{\text{fl/fl}}$ mice, nearly 40% of T cells underwent proliferation as measured by CFSE dilution. By contrast, T cell proliferation in response to $\text{Ex3}^{\text{DC-/-}}$ BMDC was dramatically impaired, as less than 5% of T cells demonstrated dilution of CFSE (Figure 5.4A). We next examined expression of the Th1 transcription factor T-bet following T cell activation by either $\text{Ex3}^{\text{fl/fl}}$ or $\text{Ex3}^{\text{DC-/-}}$ BMDC. While neither group displayed strong upregulation of T-bet, the T cells responding to $\text{Ex3}^{\text{fl/fl}}$ BMDC demonstrated significantly stronger T-bet expression than those responding to $\text{Ex3}^{\text{DC-/-}}$ BMDC (Figure 5.4B). Foxp3 expression was undetectable in T cells responding to either BMDC group (data not shown). Analysis of the cytokines in the supernatants from these co-cultures, including IL-12, IFN- γ , TNF- α , and IL-17, revealed generally inconsistent results both with regards to repeatability and agreement with CFSE results (data not shown). For instance, despite impaired T cell proliferation, supernatants occasionally revealed higher levels of IFN- γ and IL-17 following culture with $\text{Ex3}^{\text{DC-/-}}$ BMDC, though in repeat experiments this result was sometimes reversed.

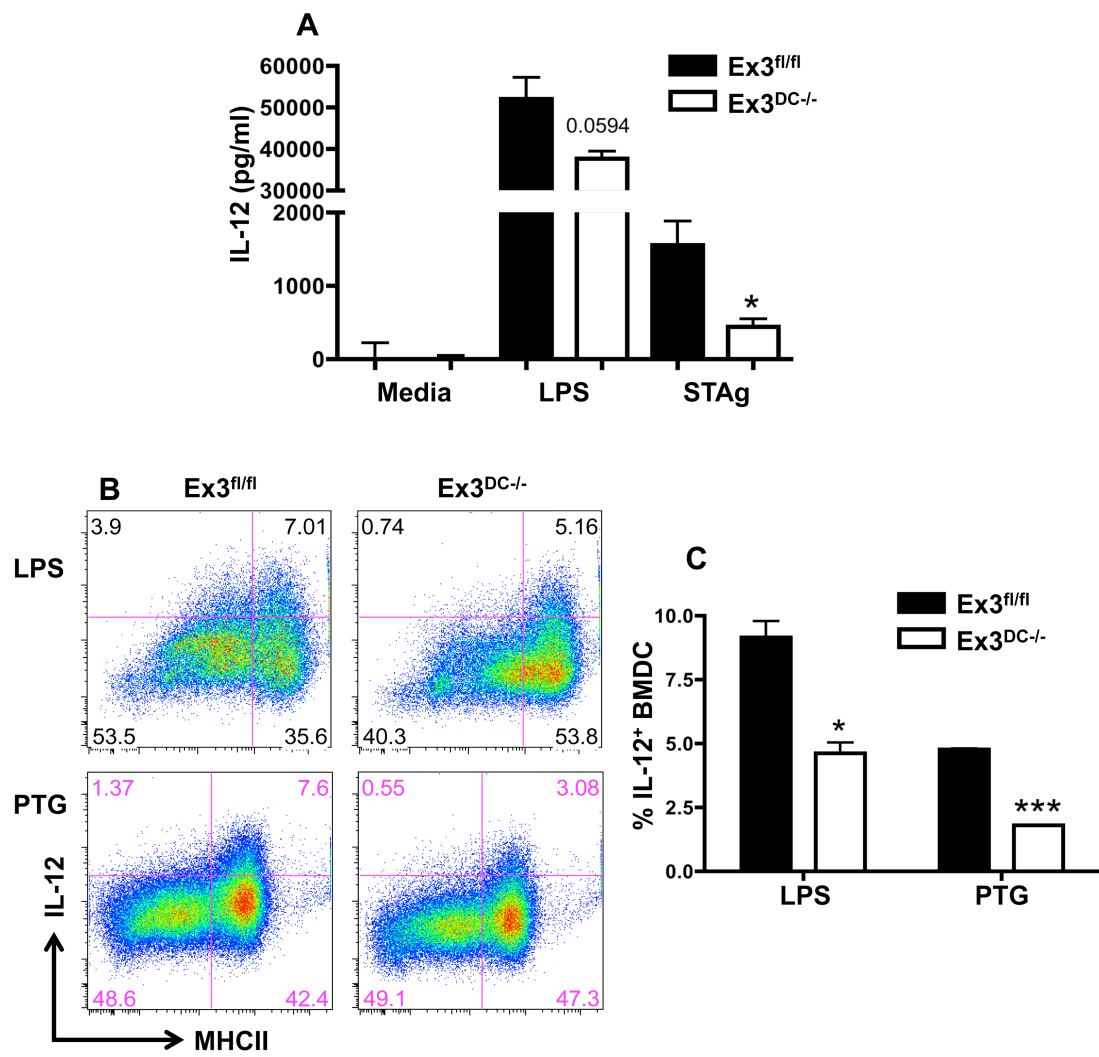


Figure 5.3. IL-12 secretion by BMDC is impaired upon constitutive β -catenin activation. (A) $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ BMDC were stimulated in vitro with LPS (100 ng/ml) or STAg (50 μ g/ml) overnight, and supernatants were assayed for IL-12p40 by ELISA. (B) $Ex3^{fl/fl}$ and $Ex3^{DC-/-}$ BMDC were stimulated with LPS or the Type II *T. gondii* strain PTG overnight, and Brefeldin-A was added for the last 5 hr of culture. Cells were then intracellularly stained for IL-12 and analyzed by flow cytometry. Shown are representative plots from individual samples. (C) Means and standard errors of IL-12 production over multiple samples.

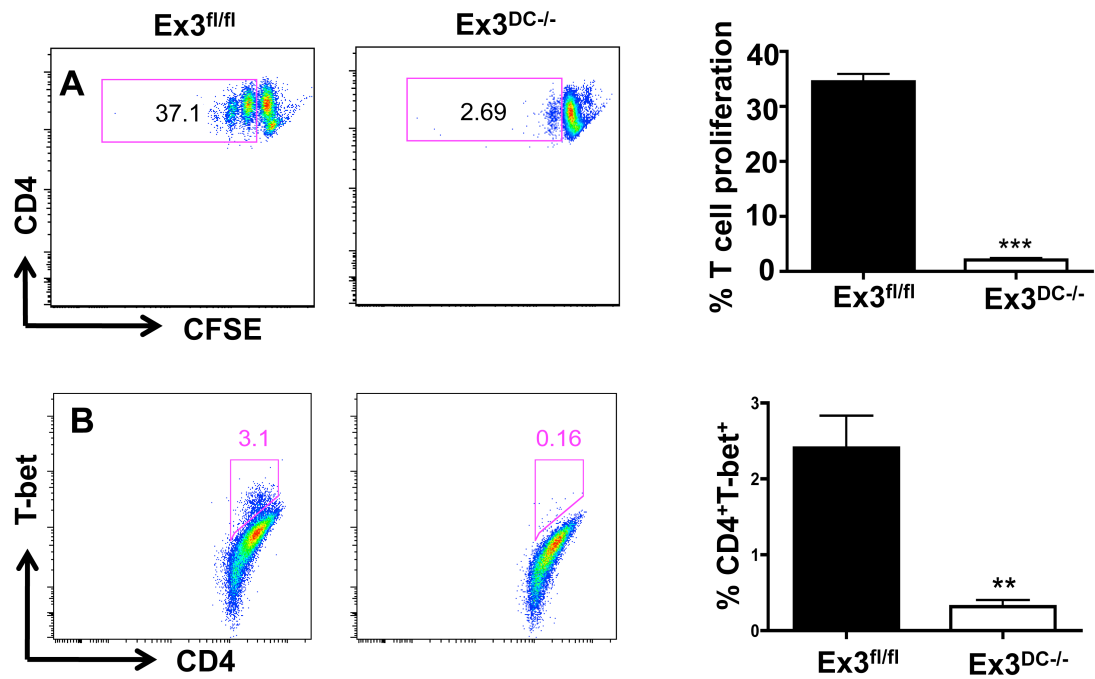


Figure 5.4. Constitutive β -catenin signaling in BMDC leads to impaired antigen-specific T cell activation. (A and B) BMDC derived from Ex3^{fl/fl} and Ex3^{DC-/-} mice were pulsed with ovalbumin (OVA) peptide and co-cultured with CFSE-labeled CD4⁺ OT-II OVA-specific T cells at a ratio of 5:1 for 96 hr. (A) Proliferation of CD4⁺ OT-II T cells as determined by CFSE dilution was measured by flow cytometry. (B) CD4⁺ OT-II T cells were intracellularly stained for T-bet expression following co-culture with Ex3^{fl/fl} and Ex3^{DC-/-} OVA-pulsed BMDC. For A and B, representative FACS plots and means and standard errors of multiple samples are shown.

Discussion

Using a transgenic approach of constitutive β -catenin activation in DC, we find that β -catenin signaling differentially affects splenic DC and BMDC. In splenic DC, β -catenin signaling led to increased phosphorylation of p38 and STAT3. Despite an early increase in ERK phosphorylation, phospho-ERK levels appeared to decline at the latest timepoint upon β -catenin activation. p38 and ERK phosphorylation have been shown to have inverse effects on IL-12 production, with p38 acting as a positive regulator and ERK as a negative regulator (19, 20). STAT3 has pleiotropic roles, as it can be activated by IL-10 or IL-6 signaling and has been shown to promote tolerogenic responses in DC (22, 25). Therefore, increased p38 and decreased ERK phosphorylation may be consistent with earlier findings of elevated IL-12 production by splenic DC with constitutive β -catenin signaling. Elevated STAT3 activation may relate to the simultaneous capacity of splenic DC with stable β -catenin to promote regulatory T cell accumulation (Cohen et al, Manuscript in Review; See Chapter 4). Furthermore, STAT3 acts downstream of Flt3L signaling and promotes the differentiation of plasmacytoid DC (pDC) through activation of the pDC transcription factor E2-2; thus, increased STAT3 phosphorylation in splenic DC by β -catenin provides further evidence of its role in promoting tissue resident DC differentiation (26, 27). Conversely, β -catenin generally provided a negative impact on BMDC maturation and function. Although MAPK and STAT phosphorylation was unaffected over a timecourse of stimulation with *T. gondii* or LPS, IL-12 secretion in response to

external stimuli, expression of costimulatory molecules, and activation of antigen-specific T cell proliferation were impaired in BMDC upon β -catenin stabilization.

While our results are in agreement with other published works that demonstrate a potential role for β -catenin in promoting tolerizing signals to BMDC, they highlight an apparent contradictory role for β -catenin in simultaneously promoting inflammatory responses in tissue resident DC. Therefore, how these in vitro findings relate to DC in the in vivo setting becomes less clear. It may be that the role of β -catenin is DC subset-specific; despite all expressing CD11c, different DC populations are driven by different transcription factors and play different roles during an immune response. For example, while $CD8\alpha^+$ DC are resident in the spleen and can therefore elicit a quick response to foreign antigen, monocyte-derived DC must be recruited into sites of inflammation from the blood and rely on chemokine production by resident cell populations.

BMDC are developmentally distinct from other DC lineages, including in vivo $CD8\alpha^+$ and plasmacytoid DC (23). In vitro derived cells are artificial by nature, and the in vivo equivalent of BMDC grown in the presence of GM-CSF remains obscure. While it has been thought that BMDC most closely resemble monocyte-derived DC/TNF- α producing inflammatory DC (Tip-DC) found in vivo due to similar surface marker expression, similar functional characteristics, and their derivation from blood monocytes through culture with GM-CSF (28, 29), this theory remains controversial, as Tip-DC still develop in mice that lack the receptor for GM-CSF (30). Furthermore,

DC-SIGN⁺ cells elicited following i.p. injection of LPS were presumed to be of monocyte origin until it was shown that they were dependent on the conventional DC (cDC)-specific transcription factor Zbtb46 and Flt3L, suggesting that they likely differentiate independently of monocytes (31, 32). Therefore, direct comparisons of in vitro BMDC results to DC results obtained in vivo are difficult and must be interpreted with caution. Regardless, many DC Wnt/ β -catenin studies have utilized in vitro derived DC as a model, and these studies have established a role for Wnt signaling in DC maturation and function through transfection or pharmacological approaches, including siRNA-mediated knockdown of β -catenin and pathway manipulation via Wnt signaling agonists or antagonists (13, 14, 33). Other studies have examined the effect of β -catenin signaling in DC using in vivo or ex vivo systems, as well (11, 12). However, the overwhelming majority of studies examining DC have demonstrated that β -catenin promotes tolerogenic responses as well as DC maturation, whether through mechanical disruption of cells or in vitro stimulation with Wnt ligands.

As described earlier, DC subsets are equipped with distinct capabilities to combat infection and are preprogrammed with different tools to accomplish their tasks. With this view, it may not be surprising that β -catenin activation yields disparate outcomes depending on the DC subset, as β -catenin target gene promoters may be more or less accessible in certain cells than in others. Furthermore, the type of stimulus may play a large role in determining the outcome of such studies, as context may affect cross-talk between β -catenin and signaling mediators, such as MAPK family members (34).

One particular discrepancy in our results is the apparent contradiction that Ex3^{DC-/-} BMDC are impaired in IL-12 production in response to stimulation yet appear to phosphorylate p38 and ERK normally (Figure 5.2B). The MAPK p38 has been shown to regulate IL-12 production by macrophages in response to *Toxoplasma* and by dendritic cells in response to other TLR ligands, while ERK has been shown to negatively regulate IL-12 production by DC (18-20, 35). The decrease in IL-12 production observed in BMDC upon β -catenin activation was fairly minimal, and it is possible that changes in p38 and ERK phosphorylation were not apparent due to sensitivity restrictions of the Western blot. Future work should examine IL-12 responses in BMDC at the mRNA level to increase sensitivity. Furthermore, it is possible that IRF8 signaling may be more important for IL-12 production in response to *Toxoplasma* than NF κ B mediated signals, as has recently been described for DC (36). Whether this is true for BMDC grown under GM-CSF conditions is not yet clear, but future work should be done to examine IRF8 upregulation in response to *Toxoplasma* in Ex3^{DC-/-} BMDC.

In conclusion, we provide further evidence of an anti-inflammatory role for β -catenin in BMDC, whereby constitutive activation of β -catenin renders BMDC unable to secrete equivalent levels of IL-12 to wild-type DC or effectively stimulate antigen-specific T cell proliferation. Considering the published reports of anti-inflammatory roles of β -catenin in BMDC, it is surprising that the anti-inflammatory phenotype of constitutively active β -catenin was not stronger, particularly with respect to

phosphorylation of downstream signaling mediators. This may indicate alternate targets of β -catenin for cytokine production or may underscore redundancy that prevents unnecessary activation or inhibition of immune responses.

Materials and Methods

Mice and infections

Mice floxed for Exon 3 of the β -catenin gene (Ex3^{fl/fl} mice) (37) were a generous gift from Björn Clausen (Erasmus University) and Aimin Jiang (Roswell Cancer Institute) and were established as breeding colonies with CD11c-cre expressing mice at the Transgenic Mouse Core Facility at the Cornell University College of Veterinary Medicine. Cre expression from all progeny was determined by PCR amplification of genomic DNA from tail snips, and Cre⁺ offspring were designated Ex3^{DC-/-} mice. C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II) mice were purchased from the Jackson Laboratory and maintained as a breeding colony at the Cornell University College of Veterinary Medicine. *Toxoplasma gondii* tachyzoites of the Type II PTG and Type I RH strains were maintained in vitro through continuous infection and lysis of human foreskin fibroblasts cultured at 37°C and 5% CO₂. BMDC were infected with tachyzoites at a ratio of 3:1 (parasites:DC), spun down at 2000 rpm for 2 min to synchronize the infection, and cultured for 24 hr.

Preparation of splenocytes

Single cell suspensions of splenocytes were prepared by crushing spleens between sterile glass slides in ice cold media and passing the resulting suspension through a 40 μ m filter. Red blood cells were then lysed with a brief incubation in ACK lysis buffer (Life Technologies).

Generation of bone marrow-derived dendritic cells

Bone marrow-derived DC were cultured as described previously (38). Briefly, femurs of $Ex3^{fl/fl}$, $Ex3^{DC-/-}$ mice were flushed with PBS and cultured for 9 days in media containing 10% fetal calf serum (Hyclone), 100 U/ml penicillin (Life Technologies), 0.1 mg/ml streptomycin (Life Technologies), 50 μ M 2-mercaptoethanol (Sigma), and 20 ng/ml GM-CSF (Peprotech). Cells were fed with 8 ml fresh media on days 3 and 6 and were given 20 ng/ml GM-CSF directly on day 8.

Western blotting

For analysis of MAPK and STAT phosphorylation, BMDC were lysed in SDS sample buffer (10^6 cells/200 μ l buffer) and proteins were separated by gel electrophoresis. Separated proteins were transferred onto a nitrocellulose membrane and blocked for 1 hr at room temperature in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk (TBST). Blots were incubated overnight in primary antibody diluted in TBST containing 5% BSA. Blots were subsequently incubated for 1 hr with anti-rabbit IgG conjugated to horseradish peroxidase-conjugated (HRP) diluted in TBST containing 5% nonfat dry milk. Blots were developed and imaged using a chemiluminescent detection system (Thermo Scientific). Anti-phospho-p38, anti-phospho-ERK, anti-phospho-STAT1, anti-phospho-STAT3, and anti-total STAT3 were purchased from Cell Signaling.

Flow cytometry

BMDC were resuspended in ice-cold PBS supplemented with 1% bovine serum albumin (BSA) and 10% sodium azide (NaN_3). 10^6 BMDC were then pelleted and resuspended in a primary antibody cocktail. Primary antibodies used for surface labeling include anti-CD11c FITC, anti-MHCII PE, anti-CD80 PerCP, anti-CD86 APC-Cy7, anti-CD4 PerCP-Cy5.5. For intracellular IL-12 staining, Brefeldin-A (eBioscience; 10 $\mu\text{g/ml}$) was added during the final 4 hr of a 24 hr culture. For all intracellular staining, cells were fixed using the FoxP3/transcription factor fixative (eBioscience) and were then incubated with primary antibodies resuspended in the FoxP3/transcription factor permeabilization buffer (eBioscience). Antibodies used for intracellular staining include anti-IL-12 PE and anti-T-bet APC.

Antigen-specific T cell proliferation

CD4^+ T cells were purified by magnetic separation from the spleens of naïve OT-II mice and were labeled with CFSE following the manufacturer's instructions (Invitrogen). Meanwhile, BMDC from $\text{Ex3}^{\text{fl/fl}}$ and $\text{Ex3}^{\text{DC-/-}}$ mice were pulsed for 4 hr with ovalbumin (OVA) peptide. OVA-pulsed BMDC (10^5) were then co-cultured with CD4^+ OT-II T cells at a ratio of 5:1 (T cell:DC) for 96 hr at 37°C . Dilution of CFSE was then assessed by flow cytometry as a measure of proliferation.

References

1. Mellman, I., and R. M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106: 255–258.
2. Kaser, A., S. Zeissig, and R. S. Blumberg. 2010. Inflammatory bowel disease. *Annu. Rev. Immunol.* 28: 573–621.
3. Xavier, R. J., and D. K. Podolsky. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448: 427–434.
4. Klaus, A., and W. Birchmeier. 2008. Wnt signalling and its impact on development and cancer. *Nat. Rev. Cancer* 8: 387–398.
5. Rao, T. P., and M. Kuhl. 2010. An Updated Overview on Wnt Signaling Pathways: A Prelude for More. *Circulation Research* 106: 1798–1806.
6. Ding, Y., S. Shen, A. C. Lino, M. A. Curotto de Lafaille, and J. J. Lafaille. 2008. Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. *Nat. Med.* 14: 162–169.
7. Biechele, T. L., R. M. Kulikaukas, R. A. Toroni, O. M. Lucero, R. D. Swift, R. G. James, N. C. Robin, D. W. Dawson, R. T. Moon, and A. J. Chien. 2012. Wnt/ β -catenin signaling and AXIN1 regulate apoptosis triggered by inhibition of the mutant kinase BRAFV600E in human melanoma. *Sci Signal* 5: ra3.
8. Masckauchán, T. N. H., C. J. Shawber, Y. Funahashi, C.-M. Li, and J. Kitajewski. 2005. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis* 8: 43–51.
9. Reya, T., A. W. Duncan, L. Ailles, J. Domen, D. C. Scherer, K. Willert, L. Hintz, R. Nusse, and I. L. Weissman. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 423: 409–414.
10. Kimura-Yoshida, C., H. Nakano, D. Okamura, K. Nakao, S. Yonemura, J. A. Belo, S. Aizawa, Y. Matsui, and I. Matsuo. 2005. Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm. *Dev. Cell* 9: 639–650.
11. Manicassamy, S., B. Reizis, R. Ravindran, H. Nakaya, R. M. Salazar-Gonzalez, Y. C. Wang, and B. Pulendran. 2010. Activation of β -Catenin in Dendritic Cells Regulates Immunity Versus Tolerance in the Intestine. *Science* 329: 849–853.
12. Oderup, C., M. LaJevic, and E. C. Butcher. 2013. Canonical and noncanonical Wnt proteins program dendritic cell responses for tolerance. *The Journal of*

Immunology 190: 6126–6134.

13. Jiang, A., O. Bloom, S. Ono, W. Cui, J. Unternaehrer, S. Jiang, J. A. Whitney, J. Connolly, J. Banchereau, and I. Mellman. 2007. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27: 610–624.

14. Zhou, J., P. Cheng, J.-I. Youn, M. J. Cotter, and D. I. Gabrilovich. 2009. Notch and wntless signaling cooperate in regulation of dendritic cell differentiation. *Immunity* 30: 845–859.

15. Jones, J. L., D. Kruszon-Moran, K. Sanders-Lewis, and M. Wilson. 2007. *Toxoplasma gondii* infection in the United States, 1999–2004, decline from the prior decade. *Am. J. Trop. Med. Hyg.* 77: 405–410.

16. Sher, A., E. Y. Denkers, and R. T. Gazzinelli. 1995. Induction and regulation of host cell-mediated immunity by *Toxoplasma gondii*. *Ciba Found. Symp.* 195: 95–104–discussion 104–9.

17. Denkers, E. Y., L. Kim, and B. A. Butcher. 2003. In the belly of the beast: subversion of macrophage proinflammatory signalling cascades during *Toxoplasma gondii* infection. *Cell. Microbiol.* 5: 75–83.

18. Kim, L., L. Del Rio, B. A. Butcher, T. H. Mogensen, S. R. Paludan, R. A. Flavell, and E. Y. Denkers. 2005. p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection. *J. Immunol.* 174: 4178–4184.

19. Kikuchi, K., Y. Yanagawa, K. Iwabuchi, and K. Onoé. 2003. Differential role of mitogen-activated protein kinases in CD40-mediated IL-12 production by immature and mature dendritic cells. *Immunol. Lett.* 89: 149–154.

20. Tang, N., L. Liu, K. Kang, P. K. Mukherjee, M. Takahara, G. Chen, T. S. McCormick, K. D. Cooper, and M. Ghannoum. 2004. Inhibition of monocytic interleukin-12 production by *Candida albicans* via selective activation of ERK mitogen-activated protein kinase. *Infection and Immunity* 72: 2513–2520.

21. Bonni, A., A. Brunet, A. E. West, S. R. Datta, M. A. Takasu, and M. E. Greenberg. 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 286: 1358–1362.

22. Melillo, J. A., L. Song, G. Bhagat, A. B. Blazquez, C. R. Plumlee, C. Lee, C. Berin, B. Reizis, and C. Schindler. 2010. Dendritic cell (DC)-specific targeting reveals Stat3 as a negative regulator of DC function. *The Journal of Immunology* 184: 2638–2645.

23. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71–82.
24. Fragoso, M. A., A. K. Patel, R. E. I. Nakamura, H. Yi, K. Surapaneni, and A. S. Hackam. 2012. The Wnt/ β -Catenin Pathway Cross-Talks with STAT3 Signaling to Regulate Survival of Retinal Pigment Epithelium Cells. *PLoS ONE* 7: e46892.
25. Park, S.-J., T. Nakagawa, H. Kitamura, T. Atsumi, H. Kamon, S.-I. Sawa, D. Kamimura, N. Ueda, Y. Iwakura, K. Ishihara, M. Murakami, and T. Hirano. 2004. IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. *J. Immunol.* 173: 3844–3854.
26. Li, H. S., C. Y. Yang, K. C. Nallaparaju, H. Zhang, Y.-J. Liu, A. W. Goldrath, and S. S. Watowich. 2012. The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development. *Blood* 120: 4363–4373.
27. Onai, N., A. Obata-Onai, R. Tussiwand, A. Lanzavecchia, and M. G. Manz. 2006. Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. *J. Exp. Med.* 203: 227–238.
28. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.
29. Serbina, N. V., T. P. Salazar-Mather, C. A. Biron, W. A. Kuziel, and E. G. Pamer. 2003. TNF/ iNOS -producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 19: 59–70.
30. Greter, M., J. Helft, A. Chow, D. Hashimoto, A. Mortha, J. Agudo-Cantero, M. Bogunovic, E. L. Gautier, J. Miller, M. Leboeuf, G. Lu, C. Aloman, B. D. Brown, J. W. Pollard, H. Xiong, G. J. Randolph, J. E. Chipuk, P. S. Frenette, and M. Merad. 2012. GM-CSF controls nonlymphoid tissue dendritic cell homeostasis but is dispensable for the differentiation of inflammatory dendritic cells. *Immunity* 36: 1031–1046.
31. Cheong, C., I. Matos, J.-H. Choi, D. B. Dandamudi, E. Shrestha, M. P. Longhi, K. L. Jeffrey, R. M. Anthony, C. Kluger, G. Nchinda, H. Koh, A. Rodriguez, J. Idoyaga, M. Pack, K. Velinzon, C. G. Park, and R. M. Steinman. 2010. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* 143: 416–429.
32. Meredith, M. M., K. Liu, G. Darrasse-Jeze, A. O. Kamphorst, H. A. Schreiber, P. Guernonprez, J. Idoyaga, C. Cheong, K.-H. Yao, R. E. Niec, and M. C. Nussenzweig. 2012. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines

the classical dendritic cell lineage. *Journal of Experimental Medicine* 209: 1153–1165.

33. Vander Lugt, B., Z. T. Beck, R. C. Fuhlbrigge, N. Hacohen, J. J. Campbell, and M. Boes. 2011. TGF- β suppresses β -catenin-dependent tolerogenic activation program in dendritic cells. *PLoS ONE* 6: e20099.

34. Guardavaccaro, D., and H. Clevers. 2012. Wnt/ β -catenin and MAPK signaling: allies and enemies in different battlefields. *Sci Signal* 5: pe15.

35. Bohnenkamp, H. R., K. T. Papazisis, J. M. Burchell, and J. Taylor-Papadimitriou. 2007. Synergism of Toll-like receptor-induced interleukin-12p70 secretion by monocyte-derived dendritic cells is mediated through p38 MAPK and lowers the threshold of T-helper cell type 1 responses. *Cell. Immunol.* 247: 72–84.

36. Raetz, M., A. Kibardin, C. R. Sturge, R. Pifer, H. Li, E. Burstein, K. Ozato, S. Larin, and F. Yarovinsky. 2013. Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to *Toxoplasma gondii* profilin. *The Journal of Immunology* 191: 4818–4827.

37. Fu, C., and A. Jiang. 2009. Generation of tolerogenic dendritic cells via the E-cadherin/ β -catenin-signaling pathway. *Immunol Res* 46: 72–78.

38. Schneider, A. G., D. S. Abi Abdallah, B. A. Butcher, and E. Y. Denkers. 2013. *Toxoplasma gondii* triggers phosphorylation and nuclear translocation of dendritic cell STAT1 while simultaneously blocking IFN γ -induced STAT1 transcriptional activity. *PLoS ONE* 8: e60215.

CHAPTER 6

Discussion

Summary of findings and future directions

The immune response to infection must be fine-tuned to efficiently eliminate invading pathogens while simultaneously preventing immune-mediated pathology. This paradigm is clearly demonstrated by infection with the protozoan parasite *Toxoplasma gondii*, as failure to express proinflammatory cytokines results in overgrowth of parasite, yet overexpression of cytokines leads to immune-mediated damage, both outcomes resulting in death of the host (1, 2). Achieving this balance involves the coordinated action of innate and adaptive immunity that together can successfully protect the host from disease. This dissertation aimed to dissect the mechanisms by which innate and adaptive immune responses develop and function to combat infection with *T. gondii*.

In **Chapter 3**, I presented data demonstrating that chemokine receptor CXCR3 expression by CD4⁺ T cells is required for effective clearance of *T. gondii* from the small intestine following oral infection. CXCR3 upregulation enabled appropriate T cell recruitment to the small intestine as well as T cell activation, as measured by IFN- γ expression. As a result of T cell-derived IFN- γ expression, inflammatory monocytes present in the gut could become primed to clear the parasite. Indeed, in the absence of this chemokine receptor, there were fewer CD4⁺ T cells in the gut and they were impaired in IFN- γ secretion, monocytes failed to upregulate markers of activation, such as iNOS and IL-12, and mice succumbed to overgrowth of parasite that was limited to the small intestine. Thus, this study underscores the importance of innate and adaptive cells cooperating to clear infection, as cytokine from T cells was required for innate immune cells to perform antimicrobial effector mechanisms.

It has become clear that chemokine-mediated trafficking is more nuanced than simple migration from blood into tissue. Rather, chemokine signaling can dictate precise locations within tissues, providing a scaffold upon which T cells and antigen presenting cells can interact to drive antigen-specific responses (3, 4). While I demonstrated a defect in trafficking of total CD4⁺ T cells to the inflamed intestine in the absence of CXCR3, it remains unclear if T cells were also impaired in activation due to impaired lymph node interactions or due to intrinsic activation defects. While CXCR3 itself is a costimulatory molecule that may provide activation signals to T cells (5), another likely possibility is that this receptor controls localization of T cells within the tissue microenvironment. Future work should examine the localization of T cells in relation to dendritic cells within the tissue by microscopy, as this will help determine how CXCR3 may be impacting intercellular interactions and resulting T cell activation.

It also remains unclear how inflammatory monocytes act as effector cells in this system. As one of the most frequently infected cells in the intestine (6), it is often assumed that monocytes are the primary effector cells to utilize immunity-related GTPases (IRG) for parasite killing. While impaired activation of monocytes was strongly associated with increased parasite burden in the absence of CXCR3, it would be interesting to formally address the role of IRG proteins in parasite killing by intestinal monocytes. This could be done through imaging experiments to monitor expression levels of IRG by infected cells in the intestine or by genetic experiments to create monocyte-specific IRG deletions and see if parasite burden is affected.

In **Chapter 4**, I made the unexpected discovery that constitutive activation of β -catenin signaling in CD11c⁺ dendritic cells (DC) (Ex3^{DC-/-} mice) led to the expansion of tissue resident DC that matched the signature of interferon regulatory factor (IRF) 8-dependent subsets, including splenic CD8 α ⁺ DC, plasmacytoid DC, and non-lymphoid tissue CD103⁺ DC. Indeed, IRF8 was strongly upregulated among these DC subsets following β -catenin accumulation. Furthermore, DC from Ex3^{DC-/-} mice were highly proinflammatory and secreted large amounts of IL-12 following stimulation with LPS or *Toxoplasma* antigen. As a result, infection of Ex3^{DC-/-} mice with a normally non-lethal dose of *T. gondii* led to severe susceptibility at the acute phase of disease.

This phenotype was surprising, as other groups have found dendritic cell-derived β -catenin to drive an anti-inflammatory phenotype, in part through the induction of Treg (7-10). Consistent with previous findings, I also observed increased Treg as a result of constitutive β -catenin signaling in Ex3^{DC-/-} mice, yet *Toxoplasma* infection results in their disappearance, rendering them non-functional (11). We believe that through this unique attribute of diminishing Treg numbers, *T. gondii* infection revealed a previously ensconced proinflammatory outcome of β -catenin signaling. This raises questions of how universal this proinflammatory phenotype is or whether it is specific to *T. gondii* infection, where expanded Treg cannot protect from disease. Future studies should aim to address this question through use of other inflammatory models, such as DSS colitis or experimental autoimmune encephalitis, both of which have been shown to require functional Treg for prevention of pathology (12, 13).

Another future goal of this project is to demonstrate a molecular interaction between β -catenin and IRF8 at the *Irf8* promoter. The results from this study suggest a direct effect of β -catenin signaling on IRF8 upregulation, as constitutive β -signaling led to increased IRF8 expression, which could be blocked at the mRNA and protein level by β -catenin inhibition. One possibility is that this is due to direct binding of β -catenin to Tcf/Lef sites within the *Irf8* promoter. Chromatin immunoprecipitation studies should be performed to address whether β -catenin or Tcf/Lef transcription factors are indeed enriched at the *Irf8* promoter.

One concern with the transgenic mouse model used in this study is the fact that CD11c is not entirely specific for dendritic cells. Thus, β -catenin may also be stabilized in monocytes, a subset of natural killer cells, and some T cells when using a CD11c-driven promoter. To avoid this issue, an interesting approach would be to delete exon 3 of β -catenin using a promoter driven by *Zbtb46*, a transcription factor that was recently found to be specific for conventional DC (14, 15). While this would exclude plasmacytoid DC as they are not conventional DC, it would allow us to focus on the role of β -catenin on CD8 α^+ DC.

In **Chapter 5**, the effect of constitutive β -catenin signaling was compared between bone marrow-derived DC (BMDC) and splenic DC. Despite a clear proinflammatory role for β -catenin signaling in splenic DC as observed in Chapter 4, this study identified an anti-inflammatory role in BMDC. As such, BMDC stimulated with LPS or *Toxoplasma* secreted decreased levels of IL-12 and failed to induce proliferation of antigen-specific T cells. Surprisingly, there were minimal changes in the

phosphorylation of downstream signaling mediators, including ERK, p38, and STAT3, upon β -catenin activation. Future studies should examine other mediators that may be affected by β -catenin signaling, including NF κ B. One potential mechanism of β -catenin-induced tolerance is through modulation of FOXO3 expression (16); thus, future studies could examine the effect of constitutive β -catenin expression on FOXO3 levels. Other pathways of DC tolerance can also be investigated, such as expression of the tryptophan degrading enzyme indoleamine dioxygenase (IDO), retinoic acid metabolizing enzymes, or inhibitory surface markers, such as CTLA-4 (17-19).

Overall, this thesis highlights the important roles of both CD4⁺ T cells and CD8 α ⁺ DC during infection with the protozoan parasite *Toxoplasma gondii* and introduces CXCR3 as a previously unidentified player in protective intestinal immunity and β -catenin as a novel regulator of DC subset differentiation. Furthermore, the data presented here underscore the complexity of immunity during microbial infection as well as at the steady state. Many questions remain unanswered, paving the way for future investigations.

References

1. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kühn, W. Müller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4⁺ T cells and accompanied by overproduction of IL-12, IFN- γ and TNF- α . *J. Immunol.* 157: 798–805.
2. Scharton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, S. Hieny, R. T. Gazzinelli, and A. Sher. 1996. In the absence of endogenous IFN- γ , mice develop unimpaired IL-12 responses to *Toxoplasma gondii* while failing to control acute infection. *J. Immunol.* 157: 4045–4054.
3. Groom, J. R., J. Richmond, T. T. Murooka, E. W. Sorensen, J. H. Sung, K. Bankert, U. H. von Andrian, J. J. Moon, T. R. Mempel, and A. D. Luster. 2012. CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4⁺ T helper 1 cell differentiation. *Immunity* 37: 1091–1103.
4. Kurachi, M., J. Kurachi, F. Suenaga, T. Tsukui, J. Abe, S. Ueha, M. Tomura, K. Sugihara, S. Takamura, K. Kakimi, and K. Matsushima. 2011. Chemokine receptor CXCR3 facilitates CD8⁺ T cell differentiation into short-lived effector cells leading to memory degeneration. *Journal of Experimental Medicine* 208: 1605–1620.
5. Groom, J. R., and A. D. Luster. 2011. CXCR3 in T cell function. *Experimental Cell Research* 317: 620–631.
6. Gregg, B., B. C. Taylor, B. John, E. D. Tait Wojno, N. M. Girgis, N. Miller, S. Wagage, D. S. Roos, and C. A. Hunter. 2013. Replication and distribution of *Toxoplasma gondii* in the small intestine after oral infection with tissue cysts. *Infection and Immunity* 81: 1635–1643.
7. Manicassamy, S., B. Reizis, R. Ravindran, H. Nakaya, R. M. Salazar-Gonzalez, Y. C. Wang, and B. Pulendran. 2010. Activation of β -Catenin in Dendritic Cells Regulates Immunity Versus Tolerance in the Intestine. *Science* 329: 849–853.
8. Jiang, A., O. Bloom, S. Ono, W. Cui, J. Unternaehrer, S. Jiang, J. A. Whitney, J. Connolly, J. Banachereau, and I. Mellman. 2007. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27: 610–624.
9. Oderup, C., M. LaJevic, and E. C. Butcher. 2013. Canonical and noncanonical Wnt proteins program dendritic cell responses for tolerance. *The Journal of Immunology* 190: 6126–6134.
10. Liang, X., C. Fu, W. Cui, J. L. Ober-Blöbaum, S. P. Zahner, P. A. Shrikant, B. E.

Clausen, R. A. Flavell, I. Mellman, and A. Jiang. 2014. β -catenin mediates tumor-induced immunosuppression by inhibiting cross-priming of CD8⁺ T cells. *J. Leukoc. Biol.* 95: 179–190.

11. Oldenhove, G., N. Bouladoux, E. A. Wohlfert, J. A. Hall, D. Chou, L. Dos Santos, S. O'Brien, R. Blank, E. Lamb, S. Natarajan, R. Kastenmayer, C. Hunter, M. E. Grigg, and Y. Belkaid. 2009. Decrease of Foxp3⁺ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* 31: 772–786.

12. Paust, S., and H. Cantor. 2005. Regulatory T cells and autoimmune disease. *Immunol. Rev.* 204: 195–207.

13. Boehm, F., M. Martin, R. Kesselring, G. Schiechl, E. K. Geissler, H.-J. Schlitt, and S. Fichtner-Feigl. 2012. Deletion of Foxp3⁺ regulatory T cells in genetically targeted mice supports development of intestinal inflammation. *BMC Gastroenterol* 12: 97.

14. Meredith, M. M., K. Liu, G. Darrasse-Jeze, A. O. Kamphorst, H. A. Schreiber, P. Guermonprez, J. Idoyaga, C. Cheong, K.-H. Yao, R. E. Niec, and M. C. Nussenzweig. 2012. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *Journal of Experimental Medicine* 209: 1153–1165.

15. Satpathy, A. T., W. KC, J. C. Albring, B. T. Edelson, N. M. Kretzer, D. Bhattacharya, T. L. Murphy, and K. M. Murphy. 2012. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *Journal of Experimental Medicine* 209: 1135–1152.

16. Watkins, S. K., and A. A. Hurwitz. 2012. FOXO3: A master switch for regulating tolerance and immunity in dendritic cells. *Oncoimmunology* 1: 252–254.

17. Wang, X. B., Z. Z. Fan, D. Anton, A. V. Vollenhoven, Z. H. Ni, X. F. Chen, and A. K. Lefvert. 2011. CTLA4 is expressed on mature dendritic cells derived from human monocytes and influences their maturation and antigen presentation. *BMC Immunol.* 12: 21.

18. Mellor, A. L., and D. H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 4: 762–774.

19. Hall, J. A., J. R. Grainger, S. P. Spencer, and Y. Belkaid. 2011. The role of retinoic acid in tolerance and immunity. *Immunity* 35: 13–22.